CHALLENGING THE GOLD STANDARD: ALTERNATIVES TO THE COLLISON FOR AEROSOL GENERATION IN RESEARCH

by

Jennifer Bowling

B.S. Biology, Coastal Carolina University, 2010

Submitted to the Graduate Faculty of

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Public Health

University of Pittsburgh

2016

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

This thesis was presented

by

Jennifer Bowling

It was defended on

April 13, 2016

and approved by

Jeremy J. Martinson, D.Phil.
Assistant Professor
Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

Robbie Mailliard, Ph.D.
Assistant Professor
Infectious Diseases and Microbiology
Graduate School of Public Health

Thesis Director: Douglas S. Reed, Ph.D.
Associate Professor
Department of Immunology
School of Medicine
University of Pittsburgh

Douglas S. Reed, Ph.D.

CHALLENGING THE GOLD STANDARD: ALTERNATIVES TO THE COLLISON FOR AEROSOL GENERATION IN RESEARCH

Jennifer Bowling, MPH

University of Pittsburgh, 2016

ABSTRACT

Animal studies to demonstrate efficacy of medical countermeasures against respiratory disease or biodefense threats require exposure of animals to aerosolized viruses and bacteria. Prior studies have shown that the choice of culture media and relative humidity in the aerosol chamber can impact the dose of infectious agent delivered to animals. Most infectious aerosol studies have involved the use of Collison jet nebulizers, which create a small, relatively monodisperse aerosol that targets the deep lung. Collison nebulizers require a relatively large volume of infectious agent, and the jets that create the aerosol may damage the agent being aerosolized. Damage resulting from the nebulizer can impact agent infectivity and virulence as well as study reproducibility. We compared the Blaustein Atomizing Module (BLAM) and the Aeroneb, a vibrating-mesh nebulizer, to the existing 'gold standard' Collison nebulizer for generation of small particle aerosols containing either a bacterium, *F. tularensis*, or a virus, influenza or Rift Valley Fever Virus (RVFV) in different exposure chambers. Aerosol performance was assessed by comparing the spray factor (the ratio between the aerosol concentration of an agent and the concentration of the agent in the nebulizer), the reduction in pathogen viability, and the aerosol efficiency (the ratio of the actual aerosol concentration to the theoretical aerosol concentration. In the Nose-only Tower (NOT), the Collison had superior aerosol performance compared to the BLAM and the Aeroneb, while the Aeroneb had superior

aerosol performance compared to the Collison in the whole-body and head-only chambers. Regression analysis revealed increased humidity was associated with improved aerosol performance of *F. tularensis*, but no environmental factors were associated with improved aerosol performance of influenza or RVFV. This data demonstrates that there is no 'one size fits all' choice of aerosol generator, and that further characterization of aerosol generators and factors that affect aerosol performance is needed to improve selection of aerosol equipment. The public health significance of this research is its contribution to the characterization of available aerosol generators to optimize aerosol experiments for a more robust experimental design for developing animal models of respiratory infections and developing therapeutics and vaccines against potential biological weapons.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

I would like to thank my mentor, Dr. Douglas Reed, for taking a chance on a Master of Public Health student. His knowledge and expertise of aerobiology and infectious diseases were greatly appreciated throughout my time working with him. I learned a lot from working with him, and he helped me greatly improve my laboratory and critical thinking skills. I am also especially grateful for the opportunity to attend several conferences to present our research, which not only aided in my understanding of the research but also helped to improve my professional presentation skills. Of course, I would also like to thank the other members of the Douglas Reed lab, especially Katie Willett. Katie was instrumental in finishing my thesis work as she helped coordinate scheduling of the aerosols and preparation of aerosol equipment. Not only was she an excellent lab member, but she was a great friend during my time working with her.

Next, I would like to thank the Amy Hartman lab and the Simon Barratt-Boyes lab for their support and opportunities to expand my thesis work to include Rift Valley Fever Virus and avian influenza. From the Amy Hartman lab, I would especially like to thank Michael Kujawa. His background with cell culture and viruses, in addition to his love and support, was greatly appreciated while working on my thesis.

I would also like to thank my other committee members, Drs. Robbie Mailliard and Jeremy Martinson. These two are excellent professors with great personalities that made coming in to class a much more rewarding experience.

1.0 INTRODUCTION

1.1 RESPIRATORY TRANSMISSION OF DISEASE

Respiratory transmission is the spread of an infection by aerosols or droplets to a susceptible individual within close proximity of the infected individual [1, 2]. As global population and international travel increases, putting more people in smaller areas, respiratory transmission poses a huge obstacle for the prevention of the spread of infectious diseases [3]. There has been much debate regarding disease transmission through aerosols [1]. Contaminated air has often been cited as the cause of many epidemics in the early history of the world [1]. With the discovery of food, water, and arthropods as vectors for disease transmission, aerosol transmission was largely rejected [1]. However, the 1917 influenza pandemic highlighted the importance of aerosols as a mode of disease transmission [4]. It is hard to prove respiratory transmission of disease, though for certain infections, such as Severe Acute Respiratory Syndrome (SARS), it is generally accepted that disease transmission is due to aerosols or airborne droplets [1, 5].

There are three main classifications for organisms spread through respiratory transmission: obligate, preferential, and opportunistic [1, 6]. Organisms that are classified as obligate transmission are only spread through aerosol deposition in the lungs, such as tuberculosis [1, 6]. Preferential transmission includes organisms that are able to cause disease

through other means of transmission but are primarily spread through aerosols, such as influenza [1, 6]. Opportunistic aerosol transmission refers to organisms that usually infect hosts through other routes, but are able to cause disease if they are transmitted through aerosols, such as Francisella or anthrax [1, 6].

A variety of microorganisms can cause disease through aerosols, though some are naturally communicable and others are artificially induced [6]. Cumulative naturally communicable respiratory infections account for the highest proportion of the global burden of disease, higher than HIV, tuberculosis, and cancer [5, 7]. Lung infections can be classified as either upper or lower respiratory infection, with lower respiratory infection having higher mortality and morbidity, and can be caused by bacteria, viruses, or fungi [6]. However, lung infections are not the only result of aerosol transmission. Smallpox and measles are spread through droplets and aerosols, but uncomplicated cases of these viruses result in maculopapular rashes rather than respiratory infections [5]. Some pathogenic agents not traditionally known for respiratory transmission can also be infectious through artificially induced aerosols. Military endeavors in the U.S. during the 20^{th} century included the development of an offensive biological weapons program with the intention of optimizing infectious agents for widespread aerosol distribution over enemy populations [6]. During this time mosquito borne viruses, such as Rift Valley Fever Virus or Venezuelan Equine Encephalitis Virus, were weaponized for aerosol dissemination [6]. The wide array of microorganisms that can be spread through aerosols and the range of clinical disease these microorganisms can cause highlight the need to study aerosol-acquired disease and the factors that can affect disease transmission.

1.2 AEROBIOLOGY

Droplet nuclei are the dried out residuals of infectious droplets less than 5um in diameter that can remain suspended in air for long periods of time [3]. These droplets are thought to be the basic mechanism for aerosol transmission of disease as they are the major component of biological aerosols [3]. Aerobiology is the study of the dispersion of aerosolized microorganisms and their byproducts and the factors that can affect this dispersion, such as relative humidity and temperature [3, 6]. This term is used in both the real world setting and the laboratory setting. Often times, real world settings of aerobiology focus on the spread of fungal spores and pollen. In research settings, aerobiology also includes the study of the effects of these airborne biological agents, such as disease pathogenesis.

Natural aerosol infection is dependent on a variety of uncontrollable factors that are largely dependent on the clinical course of disease, such as infectious dose and particle size. Such variables can contribute to differences in disease onset or pathogenesis which could therefore affect study design [8]. Thus, a controlled, experimental setting allows for a more rigorous study of aerosol-acquired disease [8].

1.2.1 FDA Animal Rule

In 2002, the Food and Drug Administration (FDA) Animal Efficacy Rule, termed Animal Rule for short, was authorized by Congress following the terrorist attacks on September 11, 2001 in response to the increased interest in the development of therapeutics and vaccines to combat potential bioterrorism [9, 10]. The Animal Rule allows the use of animals for efficacy testing of drugs or vaccines where human trials are not feasible or ethical, such as in cases where disease

incidence is too low or sporadic to effectively test or the disease causes serious health complications in humans [10, 11]. Since the implementation of the Animal Rule, there have been few new therapeutics developed. Pyridostigmine bromide and levofloxacin are FDA approved drugs for which new clinical uses have been found [9]. Cyanokit is a drug approved in Europe that has now been approved for use in the United States to treat cyanide poisoning [9, 10]. Raxibacumab is the first novel therapeutic that has been developed under the Animal Rule for the treatment of inhalational anthrax [9].

Products developed under the Animal Rule must meet four main requirements: the pathophysiology of disease and mechanism of protection of the vaccine or drug must be well defined in the animal models in order to predict human response [9-12]; the desired effect must be shown in relevant animal models to predict the response in humans, which usually requires more than one animal model; [9-12] the animal study end point must correlate with the desired effect in humans, such as reduction in death or clinical disease due to the challenge agent; and there must be sufficient pharmacokinetic and pharmacodynamics data to determine an effective dose in humans. It should be noted that clinical trials to demonstrate safety are still required.

Though the FDA Animal Efficacy Rule was designed to help promote the development of drugs and vaccines to potential bioterrorism threats, the requirements do not make the process easier [9]. Hence, they have developed guides to aid researchers in the planning, development, and testing of drugs and vaccines using the Animal Rule. To meet these requirements, the route of exposure of the agent and the route of administration of the drug or vaccine should be the same, and the dose of the challenge agent should be established to consistently reproduce disease similar in humans [10, 12]. Though ideally the challenge agent should be the same agent that causes disease in humans, the FDA recognizes that this is not always possible, such as with

smallpox. In such instances, the FDA may allow closely related agents or animal model adapted agents. Importantly, there should be enough data to prove that the disease pathogenesis is similar to human disease. Other requirements for the Animal Rule can include the following: the immune response in the animal model must be sufficiently similar to the response in humans to predict protection; the natural history of the disease should be similar to human disease; there should be a clearly defined trigger for intervention to aid in determining when to administer vaccines or drugs to humans; and there should be sufficient animals used in critical studies to perform statistical analysis [10].

1.2.2 Aerosol Study Techniques

There are three inoculation methods currently used in animal research to replicate respiratory disease transmission: intranasal, intratracheal, and aerosolization. Each method has its strengths and shortcomings, but the results from these different types of techniques are not directly comparable due to potential variances in disease pathogenesis [13].

Intranasal inoculation procedures involve gradual administration of the inoculum into the animal's nostrils [13]. Intratracheal inoculation is an invasive procedure to physically administer the inoculum through the test animal's trachea [14]. Both intranasal and intratracheal inoculation deliver a bolus of liquid to the lungs which does not mimic a true aerosol exposure. Dispersal in the lungs may not be uniform after intranasal or intratracheal inoculations, with the majority of disease at the site of inoculation [15]. Doses can be higher than are required for inhalation [14]. These factors may result in failure to achieve similar disease pathogenesis observed after aerosol exposure [13].

Inhalation exposure of aerosolized viruses or bacteria is technically challenging and requires specialized equipment to produce aerosols from an inoculum and an air flow system to deliver the aerosolized inoculum to an exposure chamber containing the test animals [14, 15]. The design and use of this type of equipment requires coordination between biological and engineering sciences to ensure successful and reproducible aerosol studies that are safe for the operator [8]. Smaller animals are not typically anesthetized while larger animals, such as nonhuman primates are anesthetized for the safety of the animal and personnel, which can alter the natural respiratory function of the animal. Aerosol exposures may also lead to deposits of infectious agent in the fur of test subjects, which could be inhaled or ingested after the aerosol infection depending on the exposure chamber used, potentially altering the desired disease course. Most experimental aerosol systems target a smaller, uniform particle size, allowing for better deposition of infectious agent in the lung, especially the lower respiratory tract. Aerosol generators are available that generate larger particle sizes to target the upper respiratory tract but these studies are the most challenging to undertake and have not been as well studied. Dosage, particle size, particle age, environmental temperature and humidity can all be measured, controlled, and analyzed to a limited degree with most experimental aerosol systems. A top-of-the-line computer-controlled system, the AeroMP, is available at the University of Pittsburgh and allows for a high degree of monitoring and control of these environmental parameters. The control of these parameters allows for the study of how these factors affect microorganisms and their aerosol infectivity.

1.2.3 Biological Aerosol Exposure Systems

The AeroMP, an automated aerosol management platform, is an exposure system used at many aerobiology research facilities around the world [16]. This system, shown in Figure 1, was developed at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) for use within a Class III Biosafety Cabinet [16, 17]. The platform was designed to withstand the repeated decontamination with paraformaldehyde gas or hydrogen peroxide vapors necessary for reuse of the aerosol equipment with different pathogens or animals [16]. The AeroMP unit electronically monitors and in some cases can control several parameters that can influence aerosol exposure, such as relative humidity, exposure duration, and sampler pressure [16, 17].

An aerosol system for exposing animals to infectious agents consists of aerosol generators, exposure chambers, and aerosol sampling devices which can include a particle sizer [16, 17]. Depending on the aerosol generator employed, compressed air may be used to generate aerosol droplets [16]. These droplets may be diluted with additional air and dried to optimal size in the mixing tube before entering the exposure chamber [16]. Simultaneously, air is pulled from the exposure chamber using a vacuum pump creating a dynamic air flow to ensure newly generated particles pass through the chamber at a steady rate [16]. Based on USAMRIID's aerosol studies, a steady-state air flow (equal input and exhaust flow rates) is established in the chamber set at a rate equal to 1 complete air change in the exposure chamber every 2 minutes. Wide varieties of aerosol sampling devices are used including impingers, gel filters, and particle sizers. In animal studies an impinger is typically used and is attached to the exposure chamber to continuously samples the aerosol throughout the experiment at a defined rate of exhaust based on

the design of the impinger and flow rate through the critical orifice [16]. This sample is then used to determine the aerosol concentration of the experiment [16].

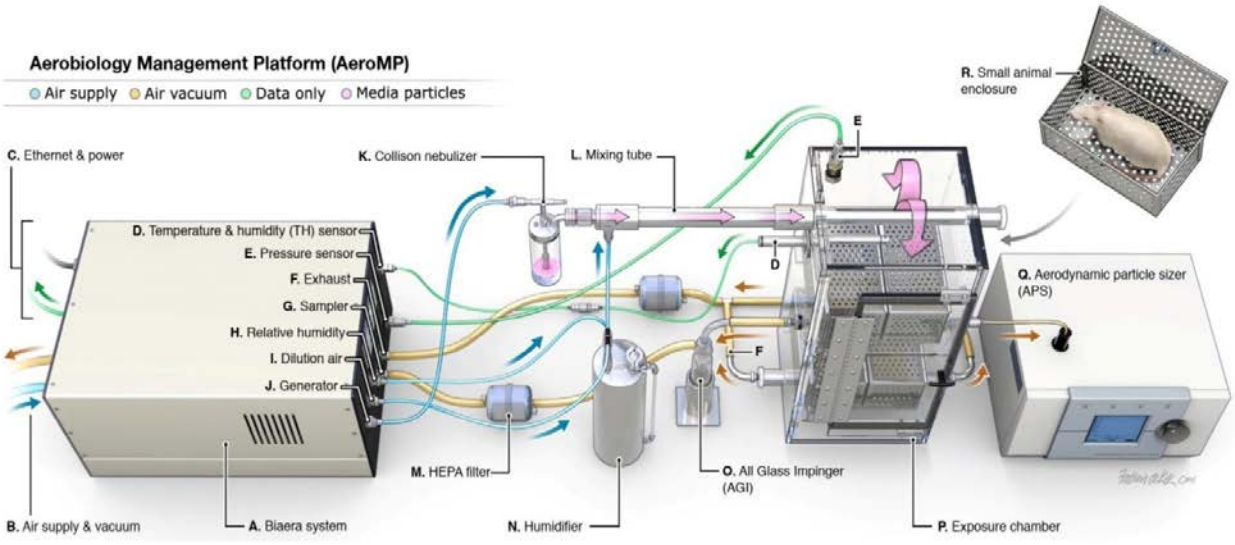

Figure 1. Schematic of the AeroMP Exposure System
Direction of airflow is indicated by the arrows. The exposure system is enclosed within a Class III Biosafety Cabinet and is controlled by software on a laptop outside of the cabinet. Compressed air enters the Biaera system, which releases a controlled flow of generator air and dilution air based on desired parameters. The generator air flows through the aerosol generator creating aerosol particles, which then enter the mixing tube where the particles are dried to optimal size by the dilution air. A vacuum pulls air through the exposure chamber to ensure a continuous flow of newly generated aerosol particles. A bioaerosol sampler and/or particle sizer may be attached to the exposure chamber. Reprinted from "ABSL-4 aerobiology biosafety and technology at the NIH/NIAID integrated research facility at Fort Detrick," by M.G. Lackemeyer, *Viruses*, *6*(1), 137-150. Copyright 2014 by P.B.Jarhling. Reprinted with permission.

1.2.3.1 Aerosol Generators

In modern infectious aerosol studies, aerosol generators employ liquid media, rather than dry powders, to produce aerosols. Dry powders containing infectious agents were commonly used during biological weapons research. Thus, there is a concern for the potential "dual use" of data gained from dry powder aerosol for bioterrorist attacks. Currently, the Collison nebulizer is the gold standard for use in aerobiology research, though there is a wide range of aerosol generators using different methodologies to produce aerosols available for use [18].

The Collison has become the standard for aerosol generation due to its low cost and easy maintenance, its relatively uniform and monodisperse particle distribution, and the small particle

sizes it generates (1 to 2 um in diameter), which are ideal for reaching the alveolar regions of the lungs in mammalian species [8]. The Collison utilizes Bernoulli's principle to create aerosols from a liquid sample [18, 19]. Bernoulli's principle states that the speed of a moving fluid, whether liquid or gas, increases as the pressure exerted by the fluid decreases [20]. Compressed air enters through the stem of the Collison and expands from the jets into the nozzle causing the liquid sample to be suctioned into the nozzle [18, 19, 21-23]. The fluid is sheared into small droplets by the air jets and is then expelled to impact against the inside of the jar [18, 19, 21-23]. Over 99.5% of this sample is refluxed back into the jar to recirculate, while the remaining aerosol droplets are carried out of the nebulizer by the spent compressed air flow [18, 19, 23]. The original version of the Collison was designed with three jets, but models with one jet, six jets, or 24 jets are now available.

Prior studies have indicated that the 3-jet model has a higher aerosol efficiency than the 1-jet model [24]. The 3-jet Collison cycles 200ml of sample every minute [19]. A 10ml sample will recirculate approximately 200 times over a ten minute aerosol. This repeated cycling places mechanical stress on the microorganisms, potentially reducing the viability or virulence and thus the aerosol efficiency [22, 25]. The potential 'battering' of the infectious agent by the Collison may result in higher doses required to cause infection and/or disease in the animal although this speculation is largely theoretical. This can make reproducibility of studies a concern if different stocks of microorganisms are used for each experiment. While the process of aerosolization will always place mechanical stress on infectious agents, aerosol generators that use less sample but have an equivalent or better aerosol efficiency in the preferred particle size range would be desired as they could better ensure reproducibility by allowing the use of the same stock of the agent throughout several studies [25, 26].

A newly developed alternative to the Collison is the Blaustein Atomizing Module (BLAM). This apparatus uses the same aerosolization method as the Collison to produce particles 0.7 to 2.5um in diameter, but it can be used in a single or multi-pass (recirculating) mode [27]. In the single pass atomization mode (SPA), the liquid media is externally introduced over a period of time into the nebulizing head where compressed air flow forces aerosol formation [27]. In multi-pass atomization mode (MPA), liquid is forced into the nebulizing head by the vacuum created by the air jets [27]. Unlike the Collison, droplets formed from atomization are expelled downwards to impact on the remaining liquid sample [27]. Small droplets impact with the liquid surface and make a 180 degree turn to be carried out of the atomizer while larger droplets collect on the surface of the liquid as they are too large make the turn [27]. Impacting the droplets over a liquid surface reduces the magnitude of force applied to the organisms compared to the Collison, hypothetically increasing the aerosol efficiency of viable organisms.

While the Collison and BLAM were both designed for use in research settings, the Aerogen® Solo Aeroneb is a single use nebulizer used in clinical settings for drug delivery. The nebulizer utilizes a palladium mesh that is 5mm in diameter and perforated with 1000 precision formed conical shaped holes [28]. Electricity is applied to the vibrational element, causing the mesh to vibrate 128,000 times per second [28]. The mesh draws the liquid through the perforations, acting as a micropump, to create a fine particle mist with particles ranging from 1um to 5um in size [28]. The clinical use Aeroneb is not designed to be autoclaved and thus cannot be reused for multiple aerosol experiments.

1.2.3.2 Exposure Chamber

The exposure chamber is the locus at which test animals are exposed to the aerosol. While choice of exposure chamber will largely be dependent on the animal species and study needs. The differing types of exposure chambers affect environment factors, such as pressure, humidity, air flow, which could affect aerosol results. Nose-only towers are designed to expose only the breathing zone of the test subjects and thus limit the potential for ingestion, dermal absorption, and other non-respiratory routes [29]. Despite this potential benefit, previous studies have indicated that even with the nose-only exposure, a variable percentage of the infectious aerosol enters the gastrointestinal tract through pulmonary clearing and subsequent swallowing [30]. Other studies indicate the restraining required for nose-only exposure increases stress levels of the test animals, as measured by corticosteroid levels, and that acclimation to the restraints does not reduce the stress of the test animals [31]. The alternatives to the nose-only chambers are whole-body and head-only chambers. As described in the name, the whole body chamber exposes the whole body of the animal and the head only chamber exposes just the head of the animal.

1.2.3.3 Aerosol Sampler

There are a variety of bioaerosol samplers available for use in aerosol research, including liquid impingers, impactors, and filters. Similar to the nebulizer, the aerosol sampler is another device in the aerosol system that can reduce viability or virulence. Thus, the choice of sampler should be considered to maximize the aerosol performance. Prior studies indicate liquid impingers cause the least amount of damage to microorganisms, leading to their widespread use in aerobiology [32]. All glass impingers (AGIs) are the most common sampler used to collect airborne microorganisms [32]. This liquid impinger is designed to mimic the airway of the

human upper respiratory tract and consists of a glass jar and a curved glass tube [32]. Air is pulled through the AGI, forcing air from the exposure chamber into the curved tube [32]. The diameter of the tube narrows, creating a critical orifice (the point at which air flows through a narrow opening at Mach one speed) where the aerosol is impacted with and collected in a liquid media [32]. The impinger sample can then be used to determine the aerosol concentration, infectious dose, or other aerosol characteristics.

1.2.4 Aerosol Performance

Aerosol performance is measured in a variety of ways; however, the method for analysis is dependent on the goal of the experiment. Clinical use nebulizers, for example, may focus more on nebulization time of drugs or ease of use. In research settings, aerosol performance may focus on particle concentration and size or biological parameters, such as culturability [22]. Determining the effect of aerosol generation on the virulence and viability of the microorganism of interest prior to exposure of animals is important to ensure accurate dose estimation and biological response [33].

The spray factor, or aerosol dilution factor, is a commonly employed method for measurement of aerosol performance. The spray factor needs to be determined for each aerosol exposure configuration and is expressed as the ratio of the aerosol concentration to the starting nebulizer concentration [8, 24, 26, 33]. This ratio allows for quality control between aerosol experiments with a specific agent as well as estimation of the starting concentration needed to reach a desired dose in an animal [8].

Particle size, expressed as aerodynamic diameter, is another method to analyze aerosol performance. The size of aerosol particles is especially important as it can affect the survival

time of the infectious agent as well as where in the respiratory tract the particle can reach [6]. Generally, particles over 10 µm deposit in the upper respiratory tract, while smaller particles are able to penetrate deeper into the lower bronchial and alveolar spaces [1, 3, 16, 32]. Microorganisms require contact with susceptible cell types to induce disease; thus, it is important in laboratory settings that nebulizers are able to produce aerosolized particles that are optimally sized to reach the desired location in the respiratory tract [6].

Another method to analyze aerosol performance that can be used to predict the dose delivered during an aerosol exposure is aerosol efficiency [34]. Aerosol efficiency characterizes the effect of artificial generation of aerosols on an organism and is expressed as the ratio of the actual aerosol concentration to the theoretical aerosol concentration [34, 35]. The actual aerosol concentration is determined from the titer collected in the bioaerosol sampler, and the theoretical concentration is determined as the amount of agent that was aerosolized by the aerosol generator [34].

1.2.5 Environmental Factors

Similar to real world situations, environmental factors such as relative humidity and temperature can vary between aerosol exposures, potentially altering efficiency of aerosol transmission and thus aerosol performance [3, 24, 26, 32, 33]. Temperature and humidity levels might alter particle evaporation, potentially affecting particle size and subsequently survival of the agent and deposition in the respiratory tract. For example, epidemiological studies have suggested that Influenza A viruses have improved transmission in low absolute humidity seen in northern climates during winter, yet transmission in tropical climates is not affected [32, 36]. Other studies examining the effects environmental factors on microbial agents in research settings

indicate *Francisella tularensis* and Rift Valley Fever Virus have improved survival following aerosol generation in higher humidity [24, 26]. These studies highlight the need to characterize fully the effects of environmental factors on aerosol performance to achieve a standardized and reproducible system for animal exposures [24, 26].

1.3 ORGANISMS

1.3.1 *Francisella tularensis*

Tularemia is caused by a small, pleomorphic, gram-negative coccobacillus, *Francisella tularensis* [37]. This disease was first clinically observed in people that consumed rabbit meat in Japan in 1837 [38]. In the United States, tularemia was described in 1911 as a plague-like illness in ground squirrels in Tulare County, California [38]. Three years later, the first clinical case in the United States was seen in a butcher [37, 38]. There are currently four identified subspecies of *F. tularensis*: tularensis, holarctica, mediasiatica, and novicida. Subspecies tularensis is highly virulent and is the dominant species found in the U.S.; subspecies holarctica is less virulent and is prevalent throughout European countries [37, 38]. Francisella is able to survive for long periods of time at low temperatures in soil, water, decaying animal carcasses, and other environments [37]. Francisella is classified as a category A biothreat agent due to its high infectivity, ease of dissemination, and ability to cause severe illness or death [38].

Every state in the United States, except Hawaii, has reported human cases of Francisella, with the majority of cases occurring in the Midwest and southwest regions [37, 39]. In Europe, tularemia is also widespread, with the highest incidence in Scandinavian countries and those of

the former Soviet Union [37]. Prior to 2015, an average of 120 cases of tularemia was reported annually in the United States; however, 175 cases of tularemia were reported in a 9-month period during 2015 [40]. This recent outbreak led to 48 hospitalizations and one death [40].

Francisella tularensis is able to infect humans through the skin, mucosal membranes, gastrointestinal tract, and the lungs, each producing a different disease pathogenesis [37, 38]. Handling infected animals, or the bite of an infected arthropod, results in an ulceroglandular disease characterized by cutaneous ulcers [38]. Ingestions of contaminated food or water may result in an oropharyngeal disease characterized by ulcers on the tonsils, pharynx, and soft pallet [38]. Pneumonic tularemia is the most acute form of disease and results from inhalation of contaminated air [37, 38]. It is characterized by hemorrhagic inflammation of the airways and systemic dissemination, with the spleen and liver being the primary location of pathological changes [37, 38]. Tularemia can be difficult to diagnose; blood tests, cultures, or PCR are used in conjunction with clinical symptoms for diagnosis [37, 40]. Though the incidence of tularemia is generally low, there is a concern that *F. tularensis* will be used in a bioterrorism event. While relatively easy to treat, rapid diagnostic tests are not widely available, so detection of intentional release relies on recognition by public health authorities [37].

Streptomycin and chloramphenicol are the licensed drugs for treatment of tularemia, though several other antibiotics used off-label have been shown to be effective [37, 38, 40]. These include gentamicin, doxycycline, ciprofloxacin, and tetracycline [37, 38, 40]. Patients treated with chloramphenicol and tetracycline sometimes relapse [38]. Antibiotic therapy is usually prolonged, but when administered properly it can reduce fatalities to about 2%. Currently there is no FDA approved vaccine for *F. tularensis*. A live attenuated vaccine derived from a virulent *F. tularensis* subspecies holarctica strain developed by the Soviet Union was

acquired and tested in the 1960s by the United States and is used to protect at-risk laboratory workers as an investigational new drug [37, 38]. This vaccine reduced the incidence of inhalation tularemia, but not ulceroglandular disease, though symptoms were often reported as milder [37, 38, 41]. Despite this reduction in laboratory cases, in a study involving human subjects, the vaccine did not protect volunteer recipients against higher aerosol challenge doses of virulent *F. tularensis* [37, 41].

1.3.2 Influenza

1.3.2.1 Seasonal Influenza

Influenza viruses are negative sense single stranded RNA, enveloped viruses of the family Orthomyxoviridae [42]. There are three types of influenza viruses, A, B, and C, but only types A and B are associated with significant disease and mortality [42, 43]. Influenza A viruses are able to infect a wide range of animal species, resulting in the seasonal antigenic drift and epidemic causing antigenic shift [42, 43]. Influenza A viruses can be subtyped based on the hemagglutinin and neuraminidase proteins on the surface of the virion [42, 43]. There are 16 HAs proteins and 9 NA proteins. Despite this diversity only H1N1, H1N2, H2N2, and H3N2 have caused pandemics [42]. Influenza B viruses do not undergo antigenic shift and thus do not have the same epidemic or pandemic potential of Influenza A viruses [42, 43].

Influenza infections peak during the winter months and primarily affect children and the elderly. In the United States, seasonal influenza results in more than 200,000 hospitalizations and 30,000 deaths annually [43]. While pandemics are much rarer, with only 11 influenza pandemics occurring during the past 300 years, the effects are much more devastating due to higher mortality rates and the lack of effective vaccines or treatment [2, 42, 43]. The 1918

Spanish Flu pandemic was responsible for 50 to 100 million deaths worldwide; the 1957 Asian Flu pandemic was responsible for more than 1 million deaths; the 1968 Hong Kong Flu pandemic was responsible for more than 700,000 deaths [42, 43].

Generally, influenza is spread person to person through respiratory droplets and aerosols, but can also be spread through fomites [42, 43]. Infectious particles must come in contact with respiratory epithelial cells in order to cause disease. People infected with influenza usually exhibit fever, a dry cough, nasal congestion, headache, sore throat, and malaise [43]. Infection is localized to the lung tissue, but in severe cases the virus can disseminate and cause encephalopathy, pericarditis, and rhabdomyolysis [43]. Influenza infections can be diagnosed with viral cultures, reverse transcriptase PCR, or antigen testing in conjunction with clinical symptoms [43]. Diagnosis can allow for viral therapy and avoid the use unnecessary use of antibiotics; however, most patients fail to seek medical care or have laboratory tests to confirm infection [43].

Several antiviral drugs are currently licensed to treat influenza in the United States [43]. Amantadine and Rimantadine block the M2 viral protein channel; oseltamivir and zanamivir are neuraminidase inhibitors [43]. These drugs are most effective when administered within 48 hours of symptoms and can reduce the duration and severity of illness, though antiviral resistance has emerged [43]. The best method to mitigate the effects of season influenza epidemics is to vaccinate against influenza [43]. Due to antigenic drift, the influenza vaccine components change annually based on the predicted strains for the upcoming influenza season [43]. Predicting the season's strain is not an exact science, and thus some years the vaccine may not be effective against influenza infection [43]. Current technology for developing influenza

vaccines is slow, hindering the ability to develop effective vaccines in the event of a poorly predicted vaccine or a pandemic as occurred in 2009 [43].

1.3.2.2 Highly Pathogenic Avian Influenza

Highly pathogenic avian influenza (HPAI) refers to strains of influenza that are extremely infectious and cause deadly disease in poultry [44]. HPAI was first reported in 1878 in Italy, but the virus was not identified as an Influenza A virus until 1955 [44]. Since the isolation of the virus, there have been 18 outbreaks of HPAI, 17 in domestic poultry and 1 in wild seabirds [44]. There have been numerous instances of direct transmission of highly pathogenic avian influenza to humans, though it is important to note the 'highly pathogenic' connotation denotes pathogenicity in poultry not humans [44]. For example, so-called low pathogenic avian influenza (LPAI) strains can be highly pathogenic in humans. One of the first outbreaks of HPAI was in 1997 in Hong Kong where 18 people were hospitalized due to H5N1 with six deaths [42-44]. While HPAIs cannot maintain sustained human-to-human transmission there is a concern that an HPAI will recombine with a human-adapted influenza virus causing to an antigenic shift that could cause a pandemic in humans [44, 45]. Another notable outbreak was in 8 family members in Sumatra, seven of which died, where the World Health Organization reported human-to-human transmission may have occurred [46].

While seasonal influenza infections are generally localized to the respiratory tract, highly pathogenic avian influenzas can cause severe systemic disease in humans. In the case of the Hong Kong outbreak of H5N1 in 1997, patients experienced fever, pneumonia, and acute respiratory distress syndrome [47]. Studies have shown that many strains of highly pathogenic avian influenza are susceptible to neuraminidase inhibitors, but resistant to the adamantine drugs [45]. Since a variety of HPAI strains have been transmitted to humans and transmission is not

sustained among humans, there is currently no vaccine to prevent HPAI infection in humans as it would be difficult to predict which particular strain of HPAI will cause an epidemic [44, 45].

1.3.3 Rift Valley Fever Virus

Rift Valley Fever Virus (RVFV) is a Phlebovirus of the family Bunyaviridae first identified during an outbreak among lambs in Kenya in 1930 [48, 49]. This negative sense, single stranded RNA virus is chiefly transmitted by mosquitoes, but can also be spread by ticks, flies, contaminated blood or tissue, and aerosols [48, 49]. RVFV primarily affects domestic animals, such as cattle, sheep, and camels, and massive abortion events, termed abortion storms, are the characteristic mark of outbreaks [26, 48]. No documentation reports human-to-human transmission of RVFV, but during the time of offensive bioweapons research, the United States was able to weaponize this virus. Due to the potential devastating effects in agriculture and the human population, RVFV is considered a high priority select agent by both the United States Department of Agriculture (USDA) and the Department of Health and Human Services [50].

Since its discovery, RVFV outbreaks among ruminants have occurred throughout Africa. The first recorded outbreak in 1930 resulted in 100,000 deaths and 500,000 abortions among sheep in South Africa [51]. Outbreaks have also occurred in Namibia, Zimbabwe, Zambia, Mozambique, Sudan, Egypt and Mauritania [51]. In September 2000, Rift Valley Fever was detected in Middle East [51]. The exact mechanism of spread of Rift Valley Fever is not known, but could be dependent on a variety factors such as mosquito ecology, climate change, and globalization. Mosquitoes capable of transmitting RVFV inhabit nearly every continent and increasing global temperature allows these mosquitoes to spread into previously virgin areas, potentially bringing Rift Valley Fever Virus to new regions [48]. Airplanes or ships may carry

infected arthropods to previously unaffected areas [48]. Infected individuals or livestock may carry the virus into new regions; [48]. if a vector competent at transmitting the virus is present in the area, the virus may become endemic to the area.

Rift Valley Fever Virus enters the human population during outbreaks of domestic ruminants [49]. The density of infected domestic animals increases the likelihood that an infected mosquito will feed on humans in close proximity. The virus is also transmitted to humans through contaminated body fluids, which can occur during birthing or slaughtering if proper protective equipment is not worn [49]. The majority of infected individuals are asymptomatic, but a small proportion exhibit disease [49]. Most symptomatic individuals show signs of influenza like symptoms, hepatitis, photophobia, or retro-orbital pain [49]. Severe cases of Rift Valley Fever infection can develop retinitis, encephalitis, and hemorrhagic fever [49]. In the absence of severe clinical manifestations, Rift Valley Fever is difficult to recognize. Laboratory diagnoses of RVF infection can be done using virus isolation, antigen detection, or reverse transcriptase PCR, though is typically limited to biocontainment facilities.

Currently there is no specific antiviral treatment for Rift Valley Fever. Treatment includes supportive care and Ribavirin, which is a nucleoside inhibitor that stops viral RNA synthesis and viral mRNA capping [49]. Public health efforts to prevent Rift Valley Fever outbreaks focus on mosquito control and the use of personal protective equipment by those with potential occupational exposure to the virus [48]. To prevent the loss of thousands of animals and potentially humans, a killed RVF vaccine and a live attenuated RVF vaccine have been used in domestic sheep and cattle in Africa and Egypt [48]. Reports indicate the live attenuated vaccine has caused vaccinated sheep to abort, indicating the virus in this vaccine can revert to its virulent form [48]. Though not FDA approved, a formalin inactivated vaccine exists for use in

humans, but it is expensive to produce, limiting its use to veterinarians and laboratory workers [48].

2.0 SPECIFIC AIMS

Reliable and reproducible aerosol experiments require a full understanding of the effects of equipment selection and environmental factors on aerosolization of pathogens and their subsequent disease. The goal of this study is to investigate potential alternatives to the Collison for aerosol generation in research use with regard to both viruses and bacteria. The Collison is the aerosol generator of choice for research studies because it is well characterized and produces high-density aerosols in the optimal size range for aerosol studies. However, the mechanism of nebulization in the Collison may cause damage to the organism [22]. The BLAM and the Aeroneb are newer aerosol generators with little data available on the ability to create bioaerosols and their effect on viability. Due to the mechanism of aerosol generation, the BLAM and Aeroneb aerosol generators are hypothesized to have improved aerosol performance compared to the Collison. Characterizations of these aerosol generators will allow for optimal selection of equipment to produce the desired response in animal models.

2.1 AIM 1: TO COMPARE THE AEROSOL PERFORMANCE OF THE BLAM AND THE AERONEB WITH THE STANDARD 3-JET COLLISON

Aerosol performance will be compared using the spray factor, the aerosol efficiency, and particle size, using one bacterium (*F. tularensis*) and two negative-strand RNA viruses (influenza and Rift Valley Fever virus) with the different aerosol generators. The goals of this aim are to:

a. Determine the optimal spray factor for each aerosol configuration using fluorescein salt as a surrogate for microorganisms as the salt cannot be damaged by aerosolization.
b. Determine the spray factor, particle size, and aerosol efficiency for each aerosol generator with different exposure chambers.

2.2 AIM 2: TO CHARACTERIZE ENVIRONMENTAL FACTORS AFFECTED BY THE CHOICE OF AEROSOL GENERATORS AND THEIR EFFECT ON PATHOGEN VIABILITY.

Relative humidity, temperature, and other aerosol parameters will be analyzed to determine if the factors are affected by choice of aerosol generator. The goals of this aim are to:

a. Determine the relative humidity and temperature ranges the different aerosol generators produce.
b. Determine if relative humidity and temperature, or other aerosol parameters, affect the viability of pathogens during aerosolization.

3.0 MATERIALS AND METHODS

3.1 BIOSAFETY

All aerosol experiments for this study were performed in a class III biological safety cabinet within the dedicated Aerobiology Suite in the University of Pittsburgh Regional Biocontainment Laboratory (RBL). Powered air purifying respirators (PAPRs) were worn for respiratory protection during work with H5N1 and RVFV, and all work with these viruses was conducted at BLS3 conditions in class II biosafety cabinets in the RBL, using Vesphene IIse (diluted 1:128, Steris Corporation, cat. #646101) for disinfection. Spatial and temporal separation was maintained between H5N1 and all other infectious agents. Work with *F. tularensis* LVS strain and seasonal influenza was conducted at BSL2+ conditions in a class II biosafety cabinet using 10% bleach or Vesphene IIse (1:128) for disinfection.

3.2 FRANCISELLA TULARENSIS

All *Francisella tularensis* aerosols used live attenuated vaccine (LVS) strain *Francisella tularensis* obtained from Jerry Nau. LVS stock was grown on August 7, 2008 and stored at -80°C.

3.2.1 Culture Methods

LVS was grown on Cysteine Heart Agar (CHA; BD Difco™, cat. #247100 and BD BBL™, cat. #212392) for two days at 37°C, 5% CO_2. Broth cultures were set up with 0.5mls of bacteria suspended in 1X Phosphate Buffered Saline (PBS) at an optical density of 0.06 to 0.08 and 24.5mls of Brain Heart Infusion (BHI; BD BBL™, cat. #211059) broth, supplemented with 2.5% ferric pyrophosphate and 1.0% L-Cysteine hydrochloride, in a filter top, baffle bottomed flask. The cultures were incubated at 37°C in an orbital shaker at 200rpm and harvested between 15 to 18 hours to ensure the bacteria was in the logarithmic growth phase [24].

3.2.2 Concentration determination

Concentration of LVS was determined by colony counts on CHA. Tenfold serial dilutions of LVS aerosol samples were prepared in 1X PBS. A multichannel pipette was used to dispense 10µl of 5 consecutive dilutions to the top of a CHA plate held at a 45° angle. The samples were allowed to drip down the plate, ensuring no sample paths crossed. The plates were then placed agar side up to all the samples to air dry. All plated dilutions were repeated in duplicate or triplicate. Once dry, the CHA plates were inverted and incubated at 37°C/5% CO_2 for two days. Dilutions with 15-100 colonies were chosen for titer calculations.

3.3 INFLUENZA

Influenza A/PR/8/34 (H1N1), Influenza A/Syd/5/37 (H3N2), Influenza A/Ca/4/09 (H1N1), and Influenza A/Vietnam/1203/04 (H5N1) were used for aerosol experiments. Influenza A/PR/8/34 was obtained from Rich Webby and Influenza A/Syd/5/37 was obtained from Dr. Mickey Corb; both were propogated in MDCK cells. Influenza A/Ca/4/09 was obtained from Biodefense and Emerging Infectious Resources and propagated in MDCK cells or chicken eggs. H5N1 stock was propagated in MDCK cells or chicken eggs and obtained from the Centers for Disease Control and Prevention. Temporal and spatial separation of all strains of influenza was maintained throughout the experiments.

3.3.1 Determining Concentration

Influenza virus titer was determined by standard TCID50 or plaque assay using Madin-Darby Canine Kidney Epithelial (MDCK) cells. MDCK cells (ATCC CCL-34) were cultured in DMEM-10 (Dulbecco's Modified Eagle's Medium with 10% heat inactivated fetal bovine serum, 1% penicillin/streptomycin, and 1% HEPES). Cells were passaged at 90% confluency using 1X PBS to wash the cells and 0.25% trypsin/EDTA to dissociate the cells from the tissue culture flask. Trypsin/EDTA was neutralized with DMEM-10 at a ratio of 1:5, and cells were passaged into a new flask with fresh DMEM-10. Cultured cells were incubated at 37°C/5% CO_2 between passages.

For TCID50s, MDCK cells were seeded into a flat bottomed sterile 96 well plate at a concentration of 3.0e105 cells/ml and incubated at 37°C/5% CO_2 overnight until 95% confluent. Tenfold serial dilutions of influenza aerosol samples were prepared in Viral Growth Medium

(VGM; Dulbecco's Modified Eagle's Medium, 2.5% of 7.5% bovine serum albumin fraction V, 1% penicillin/streptomycin, 1% HEPES buffer, and 0.1% TPCK trypsin). Medium was removed from cells and replaced with 150µl of VGM. Five wells in one row were infected with 100µl each of one dilution. This was repeated for each row, with the first row receiving undiluted sample and the last row receiving the lowest dilution. Each well of the 6th column received 100µl of VGM to act as a control. The plates were incubated at 37°C/5% CO_2 for 48 hours. Cells were then examined under a microscope for cytopathic effect (CPE) as compared to the controls. Each well was scored as positive or negative for CPE. Viral titers were then calculated using the method described by Reed and Muench [52]..

For seasonal influenza plaque assays, MDCK cells suspended in DMEM-10 were seeded into 6-well tissue culture plates and incubated at 37°C/5% CO_2 overnight until 95% confluent. Tenfold serial dilutions of influenza aerosol samples were prepared in VGM. Medium was removed from the cells and 500µl of inoculum was added to each well; each dilution was repeated in duplicate. Plates were incubated at 37°C/5% CO_2 for 1 hour. After incubation, inoculum was removed from the wells and a 1% nutrient overlay (2X Modified Eagle Medium, BSA, penicillin/streptomycin, 2% agarose) was added. The overlay was allowed to polymerize and then the plates were incubated at 37°C/5% CO_2 for 3 to 5 days depending on strain. Plates were fixed with 2mls of 37% formaldehyde for at least 2 hours. The formaldehyde was drained and the agar plugs were removed from the wells. The cell monolayer was stained with a 0.1% crystal violet stain to visualize plaques. Wells with 15 to 100 plaques were counted for titer calculations.

H5N1 plaque assays were performed in the same manner as seasonal influenza plaque assays with the following changes: 800µl of inoculum was used instead of 500µl; following the

addition of inoculum, the plates were incubated at 4°C for 10 minutes, then incubated at 37°C/5% CO2 for 50 minutes; a 0.9% nutrient overlay was used instead of a 1.0% nutrient overlay.

3.4 RIFT VALLEY FEVER VIRUS

Rift Valley Fever Virus (RVFV) aerosols used strain ZH501, an isolate from patient 501 during the 1977 outbreak. Recombinant RVFV was obtained from Barry Collins (CDC, Ft. Collins, CO) and propagated in Vero E6 cells.

3.4.1 Sample Preparation and Concentration Determination

All Rift Valley Fever Virus samples and media for AGI-30s were prepared by the Amy Hartman laboratory group. RVFV titer was determined by standard plaque assay using Vero E6 cells. All Rift Valley Fever Virus plaque assays were completed by Dr. Amy Hartman's laboratory.

3.5 AEROSOL SETUP

3.5.1 Aerosol Exposures

The AeroMP from Biaera Technologies (Hagerstown, MD) was used to monitor, record, and modulate aerosol parameters during aerosol experiments. Aerosol experiments consisted of three to five runs of one aerosol setup with one agent, ten minutes in length. These experiments were

repeated in at least triplicate on different days to account for normal variance between aerosol experiments. The airflow parameters of the aerosol experiments were programmed to do one complete air change in the exposure chamber every two minutes.

3.5.2 Aerosol Generators

Three aerosol generators were used in these studies: the Collison, the single use Aeroneb, and the BLAM (Figure 2). The Collison was calibrated using the Gilibrator 2 (Sensidyne™ Gilian™) before each aerosol to ensure an air flow of 7.5 ± 0.25 L/min; the BLAM and Aeroneb did not require air flow checks. The Collison and BLAM were autoclaved at 121°C for 30 minutes prior to each aerosol experiment; the Aeroneb Solo is a single use device and is shipped in sterile packaging. The BLAM was assessed in single-pass atomization mode using a programmable syringe pump (Model no. NE-1000, New Era Syringe Pump, Inc). The syringe pump was programmed to dispense sample at a rate of 1ml per minute into the BLAM.

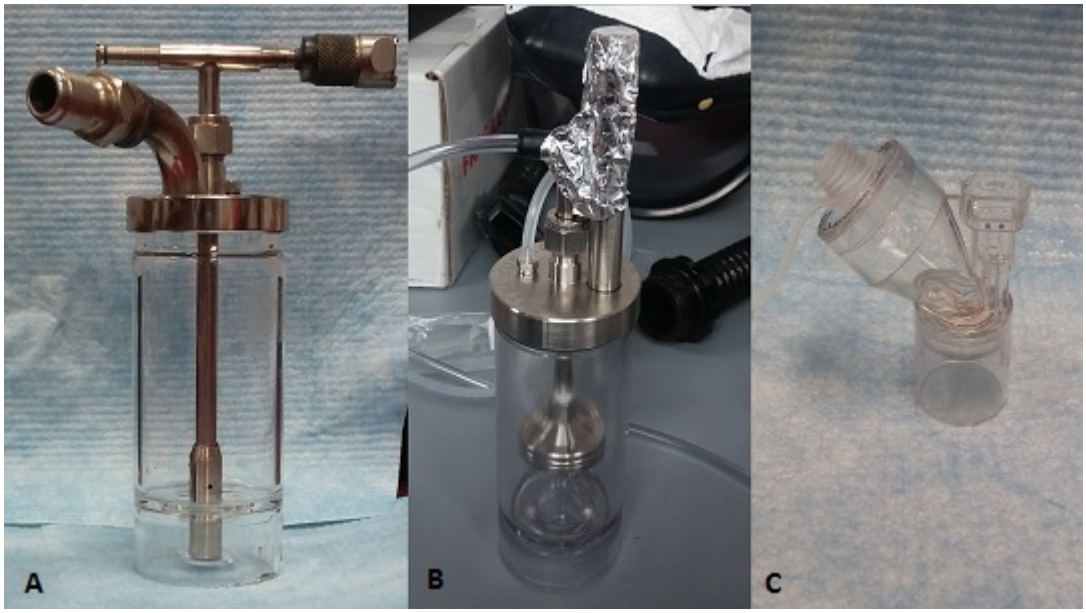

Figure 2. Aerosol Generators
Image of the three aerosol generators compared in this experiment: A) The Collison, B) The BLAM, C) Aeroneb.

Table 1 Flow Rate and Pressure of Aerosol Generators

Aerosol Generator	Flow Rate	Pressure
Collison	7.5 L/min	26-30 PSIG
BLAM	8.0 L/min	13-15 PSIG
Aeroneb	0.0 L/min	0 PSIG
*PSIG per square inch gauge		

On the day of the aerosol experiment, the overnight LVS broth culture was harvested and diluted with additional BHI to reach a total volume of 45 or 65ml. Frozen (-80°C) seasonal influenza stock was diluted 1:10 or 1:100 with VGM. H5N1 stock was diluted with VGM to reach the necessary concentration to reach a specific target dose. Rift Valley Fever Virus was diluted 1:10 or 1:100 with equal parts D2 (Dulbecco's Modified Eagle's Medium with 2% fetal

bovine serum, 1% penicillin/streptomycin, 1% L-glutamine) and antifoam solution (40µl antifoam, 80µl glycerol, and 100µl HEPES) for aerosol experiments.

3.5.3 Aerosol Sampling

Bioaerosol sampling was achieved using the all glass impinger-30 (AGI-30) calibrated with the Gilibrator to ensure an airflow of 6.0 ± 0.25 L/min (figure). For LVS aerosols, 10ml of BHI broth and 40µl of antifoam A (Fluka, cat. #10794) was added to each AGI-30. For influenza aerosols, 10ml of VGM and 80µl of antifoam was added to each AGI-30. For Rift Valley Fever Virus, 10ml of D2 and 40µl of antifoam was added to each AGI-30. Particle size was determined using the Aerodynamic Particle Sizer Spectrometer (APS^{TM}) from TSI, Inc. (Shoreview, MN).

3.5.4 Exposure Chambers

Four different exposure chambers were used in these studies shown in Figure 3: the nonhuman primate head-only chamber (NHP HO), the ferret whole-body chamber (FWB), the rodent whole-body chamber (RWB), and the rodent nose-only tower (NOT). Table 2 summarizes the volume of the exposure chambers and the programmed flow rate used for aerosol experiments.

Figure 3. Exposure Chambers
Image of three of the different exposure chambers used in this experiment: A) NOT, B) FWB chamber, and C) NHP HO chamber. The RWB chamber (not shown) is similar in design to the FWB chamber but has a smaller volume.

Table 2 Chamber Air Volumes and Aerosol Flow Rate

Chamber	Volume	Flow Rate
Rodent Nose-only Tower	12 L	8.0 L/min
Rodent Whole-body Chamber	39 L	19.5 L/min
Ferret Whole-body Chamber	44 L	22.0 L/min
NHP Head-only Chamber	32 L	16.0 L/min

The Collison and Aeroneb were compared for aerosolization of LVS in the nose-only tower and the rodent whole-body chamber. For influenza, the Collison and the Aeroneb were compared in the nose-only tower, the ferret whole-body chamber, and the nonhuman primate head-only chamber. Aerosol performance of Rift Valley Fever Virus between the Collison and the Aeroneb was compared in the rodent whole-body chamber. The BLAM was designed for use with the NOT, and thus was only tested with LVS and seasonal influenza.

3.6 AEROSOL PERFORMANCE

3.6.1 Spray Factor and Aerosol Efficiency

Aerosol performance between nebulizers was compared using the spray factor (SF) and aerosol efficiency. Aerosol generator and AGI-30 titers were used for SF and aerosol efficiency calculations. The SF is the ratio of the aerosol concentration to the starting aerosol generator

concentration. Aerosol efficiency is the ratio of the true aerosol concentration to the theoretical aerosol concentration. SF and aerosol efficiency were calculated as follows:

$$SF = \frac{[actual\ aerosol]}{[aerosol\ generator]}$$

$$Aerosol\ efficiency = \frac{[actual\ aerosol]}{[theorerical\ aerosol]}$$

where:

$$[actual\ aerosol] = \frac{[AGI] * VAGI}{QAGI * Texp}$$

$$[theoretical\ aerosol] = \frac{[aerosol\ generator] * Vnebulized}{Qexp * Texp}$$

In the equation V is volume, Q is flow rate, and Texp is the time of the exposure.

3.6.2 Log Reduction in SF Due to Loss of Viability

Fluorescein salt (Sigma, cat. #F6377) was added to some aerosol experiments to be used as an indicator of ideal spray factor given natural loss. In fluorescein salt experiments, 0.1mg of fluorescein salt was dissolved in 1ml of ddH2O. Log reduction was then calculated as follows:

$$Log\ Reduction = log10\ (\frac{Fluorescein\ SF}{Pathogen\ SF})$$

3.7 STATISTICAL ANALYSIS

GraphPad Prism® 6 was used to create all spray factor figures and to perform two-sided Mann-Whitney U tests to compare the spray factor and aerosol efficiency between nebulizers. This nonparametric test was chosen due to the non-normal distribution of results and the high frequency of outliers.

STATA was used to perform a multiple linear regression to determine if collected environmental parameters, specifically chamber relative humidity, temperature, and sampler pressure, affect aerosol performance. Temperature and humidity were included since prior studies indicate aerosol performance of certain pathogens are affected by these parameters. Sampler pressure (pressure in the AGI) was included due to observations regarding aerosol performance potentially related to sampler pressure.

4.0 RESULTS

4.1 AEROSOL PERFORMANCE

The ‘gold-standard’ Collison nebulizer may be overly harsh when aerosolizing pathogens. This could raise the nebulizer concentration needed to achieve a desired challenge dose in animal models and may affect virulence and host response, raising the dose required to cause morbidity and mortality in animal models. Other nebulizers might achieve better viability of pathogens in experimental aerosols, thereby lowering the concentration needed to achieve a desired dose in animal challenge studies. The goal of these studies was to compare performance of two alternative aerosol generators, the BLAM and the Aeroneb, to the Collison. Both produce particles in the same general size range as the Collison (between 1-5 μm MMAD), and such aerosol particles should be capable of reaching the deep lung. Aerosol performance of the aerosol generators was evaluated using spray factor (SF) and aerosol efficiency (AE). Aerosol performance of these nebulizers was further evaluated in different exposure chambers and using both viruses and bacteria to evaluate whether differences seen were specific to a particular chamber or pathogen.

4.1.1 *Francisella tularensis*

The different nebulizers were first assessed by aerosolizing *F. tularensis* (LVS) into two different aerosol chambers, the nose-only tower (NOT) and rodent whole body chamber (RWB). Aerosol exposures were ten minutes in length, and aerosol samples were collected in 10mls of BHI in AGIs. Nebulizer and AGI concentrations were determined by plating serial dilutions on CHA plates. Table 3 summarizes the SF results of each aerosol generator in each exposure chamber. In the NOT, the Collison had the highest average SF (6.38E-07) compared to the BLAM (1.15E-07) and the Aeroneb (1.01E-08). The coefficient of variation (CV) was highest in the BLAM (78.8%), followed by the Collison (62.1%) and the Aeroneb (27.8%), indicating the Aeroneb performance had better reproducibility in the NOT. In the RWB chamber, the Aeroneb had the higher average SF (4.70E-08) compared to the Collison (2.98E-09). The CV for the Collison was 99.8%, while it was 153.2% for the Aeroneb. Figure 4 shows the spray factors of LVS in the NOT and the RWB using the BLAM, Aeroneb, and Collison. Based on a two-sided Mann-Whitney U test, the SF in the NOT was significantly different between the Collison and the BLAM (p-value<0.0001) and the Collison and the Aeroneb (p-value<0.0001), but the mean SF indicates the Aeroneb and the BLAM did not outperform the Collison in Aerosol generation. In the RWB chamber, the SF was significantly different between the Collison and the Aeroneb (p-value$=0.0004$), with the Aeroneb achieving better aerosol performance.

Table 3 Summary of SF Results for LVS Aerosols

	NOT			RWB	
	Collison	BLAM	Aeroneb	Collison	Aeroneb
Mean	6.381E-07	1.152E-07	1.009E-08	2.98E-09	4.70E-08
SD	3.961E-07	9.074E-08	2.807E-09	2.98E-09	7.21E-08
CV	62.07%	78.77%	27.82%	99.80%	153.21%

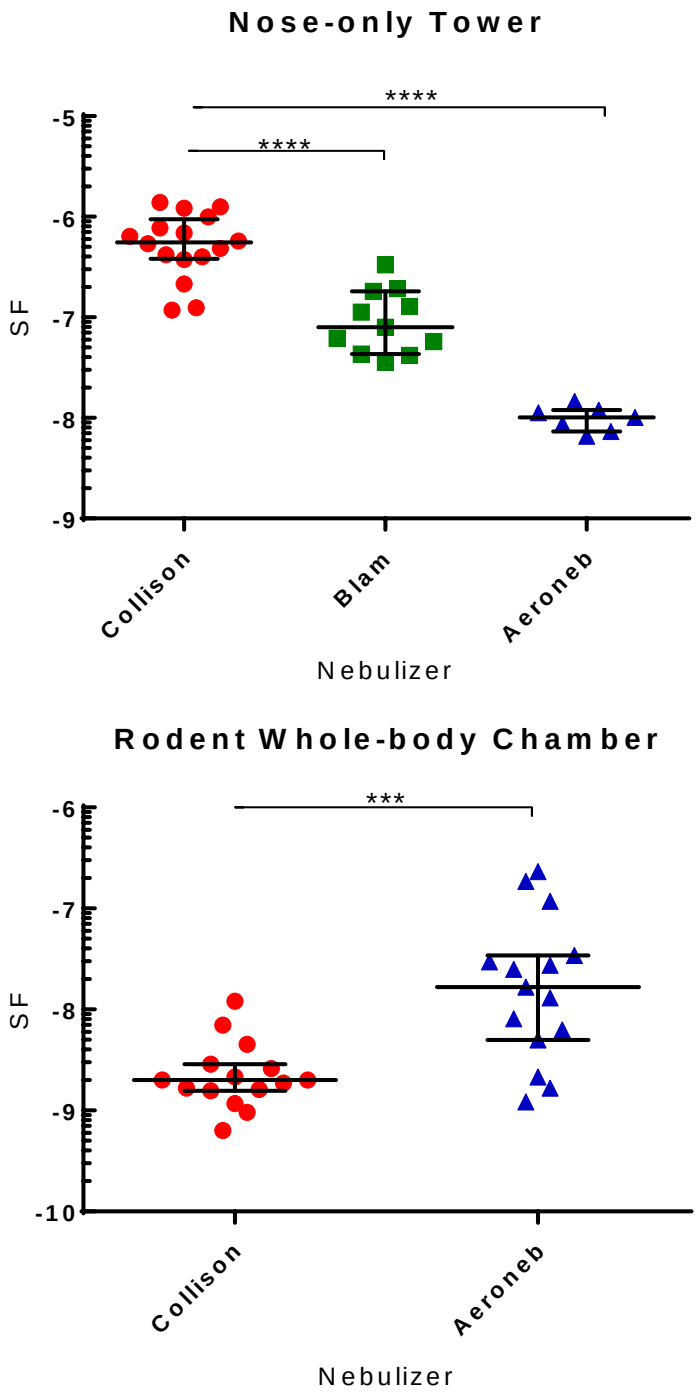

Figure 4. Spray Factor of LVS is Higher with the Collison in the NOT and Higher with the Aeroneb in the RWB Chamber

The Collison is represented by red symbols, the BLAM by green symbols, and the Aeroneb by blue symbols. Error bars represent the median and interquartile range. In the NOT, the SF is significantly different between the Collison and the BLAM (p-value<0.0001) and the Collison and the Aeroneb (p-value<0.0001). In the RWB chamber, the SF was significantly different between the Collison and the Aeroneb (p-value=0.0004).

Fluorescein salt was added to some aerosol experiments to determine the theoretical spray factor possible if no damage occurred to organisms. Table 4 summarizes the log reduction in SF between the fluorescein salt and LVS. In the NOT, the Collison resulted in a 1.44 log reduction in LVS SF (compared to fluorescein), the BLAM resulted in a 2.16 log reduction in LVS SF, and the Aeroneb resulted in a 2.85 log reduction in SF. In the RWB chamber, the Collison resulted in a 3.61 log reduction in SF, while the Aeroneb resulted in a 3.08 log reduction. Figure 5 shows a graphical representation of fluorescein SF compared to LVS SF with each aerosol generator in the NOT and the RWB chamber. The difference in SF between Fluorescein and LVS was significant for the Collison (p-value=0.0079), BLAM (p-value<0.0001), and Aeroneb (p-value=0.0006) in the NOT and for the Collison and Aeroneb in the RWB chamber (p-value<0.0001 for both).

Table 4 Log Reduction in Spray Factor for LVS in the NOT and the RWB Chamber

NOT			
Aerosol Generator	Median Fluorescein SF	Median LVS SF	Log Reduction
Collison	1.50E-05	5.40E-07	1.44
BLAM	8.35E-06	5.74E-08	2.16
Aeroneb	7.08E-06	1.01E-08	2.85
RWB			
Aerosol Generator	Fluorescein Median SF	LVS Median SF	Log Reduction
Collison	8.14E-06	2.01E-09	3.61
Aeroneb	9.74E-06	8.12E-09	3.08

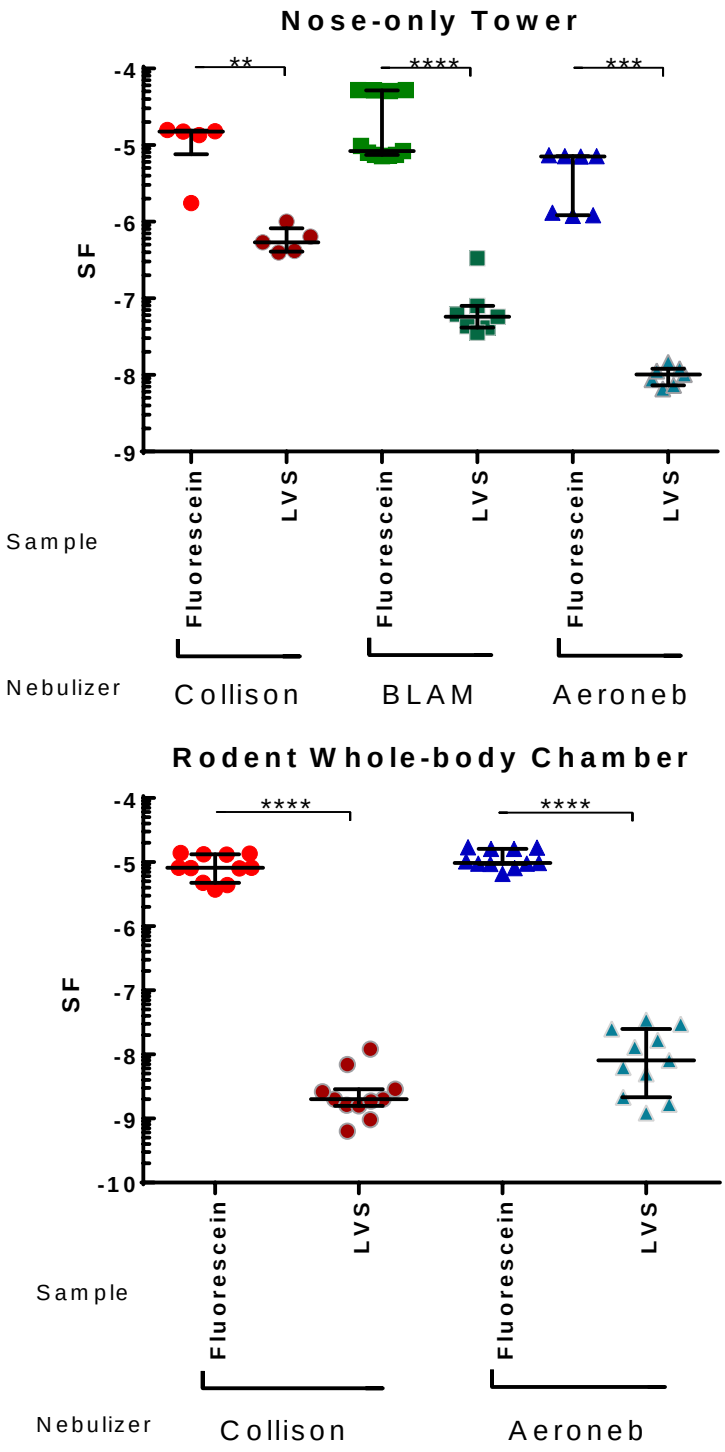

Figure 5. Significant Decrease in LVS Viability with All Aerosol Generators
Red symbols represent the Collison, green symbols the BLAM, and blue symbols the Aeroneb. Error bars represent the median and interquartile range. Fluorescein salt SF represents the maximum achievable SF for each aerosol generator, while LVS SF represents the actual SF achieved using a live bacterium, LVS.

Aerosol efficiency of each aerosol generator is summarized in Table 5. In the NOT, the Collison had the highest efficiency (1.598%), followed by the BLAM (0.639%) and the Aeroneb (0.035%). In the RWB chamber, the Aeroneb (0.108%) had the higher aerosol efficiency than the Collison (0.012%). The Aeroneb aerosol efficiency was significantly different from the Collison in both the NOT and the RWB chamber (p-value<0.0001).

Table 5 Aerosol Efficiency of LVS in the NOT and RWB Chamber

NOT		
Aerosol Generator	Median Aerosol Efficiency	P-value
Collison	1.598%	
BLAM	0.639%	**0.031**
Aeroneb	0.035%	**<0.0001**
RWB		
Collison	0.012%	
Aeroneb	0.108%	**<0.0001**
*Based on a two-sided Mann-Whitney U test **Signficant results in bold		

4.1.2 Influenza

Aerosol performance of seasonal influenza A viruses was assessed in the rodent nose-only tower (NOT), the ferret whole-body (FWB) chamber, and the nonhuman primate head-only (NHP HO) chamber. Differences in SF between different Influenza A subtype viruses were also evaluated

(Figure 6). There was no significant difference between the SF of H3N2 and H1N1 with the Collison in both the FWB and the NHP HO chambers. However, when using the Aeroneb there was a significance difference between the SF of H3N2, H1N1, and H5N1 (p-value=0.0005) in the NHP chamber, based on a Kruskal-Wallis test. Dunn's multiple comparison analysis reveals the SF of H3N2 is significantly different from the SF of H1N1 and H5N1, but there is no difference between the SF of H1N1 and H5N1. Due to limitations in the H5N1 stock available, I did not evaluate aerosolization of H5N1 with the Collison in either the NHP or the FWB chamber.

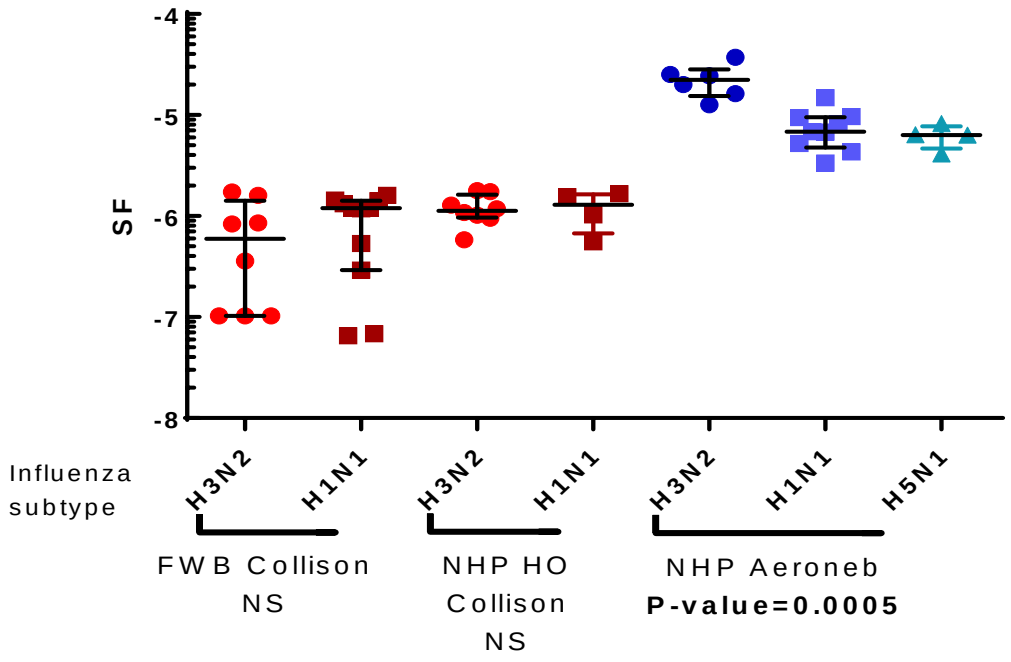

Figure 6. Spray Factor of Influenza is Similar Between Strains with the Collison, but is Significantly Different with the Aeroneb
Several subtypes of Influenza A were used to compare aerosol generators. The Collison is represented by red symbols and the Aeroneb by blue symbols. There is no significant difference between H3N2 and H1N1 with the Collison in either the FWB or the NHP HO chambers. There is a significant difference between Influenza A subtypes with the Aeroneb in the NHP HO chamber.

The SF results for the influenza aerosols is summarized in Table 6. The Collison (6.56E-06) resulted in the highest average SF for influenza in the NOT, followed by the Aeroneb (5.97E-06) and the BLAM (5.03E-07). The CV of was highest for the Aeroneb (209.8%), followed by the Collison (108.8%) and the BLAM (53.3%) in the NOT. The average SF for influenza was higher for the Aeroneb (5.30E-06) than the Collison (1.02E-06) in the FWB chamber. The CV was lower for the Aeroneb (47.9%) than the Collison (54.5%) in the FWB. In the NHP HO chamber, the average SF was higher in the Aeroneb (9.70E-06) than the Collison (1.19E-06). The CV was 52.3% for the Aeroneb and 34.0% for the Collison in the FWB.

Figure 7 shows the impact of choice of aerosol generator on the SF of Influenza A. In the NOT, the SF of Influenza for both the BLAM and the Aeroneb are significantly different from the Collison based on a two-sided Mann-Whitney U test (p-value<0.0001 and p-value=0.0145, respectively). The SF of Influenza for the Aeroneb is significantly different from the Collison in the FWB (p-value<0.0001) and NHP HO (p-value<0.0001) chambers.

Table 6 Summary of SF Results for Influenza Aerosols

	NOT			**FWB**		**NHP HO**	
	Collison	BLAM	Aeroneb	Collison	Aeroneb	Collison	Aeroneb
Mean	6.56E-06	5.03E-07	5.97E-06	1.02E-06	5.30E-06	1.19E-06	9.70E-06
SD	7.14E-06	2.68E-07	1.25E-05	5.57E-07	2.54E-06	4.06E-07	5.07E-06
CV	108.8%	53.3%	209.8%	54.5%	47.9%	34.0%	52.3%

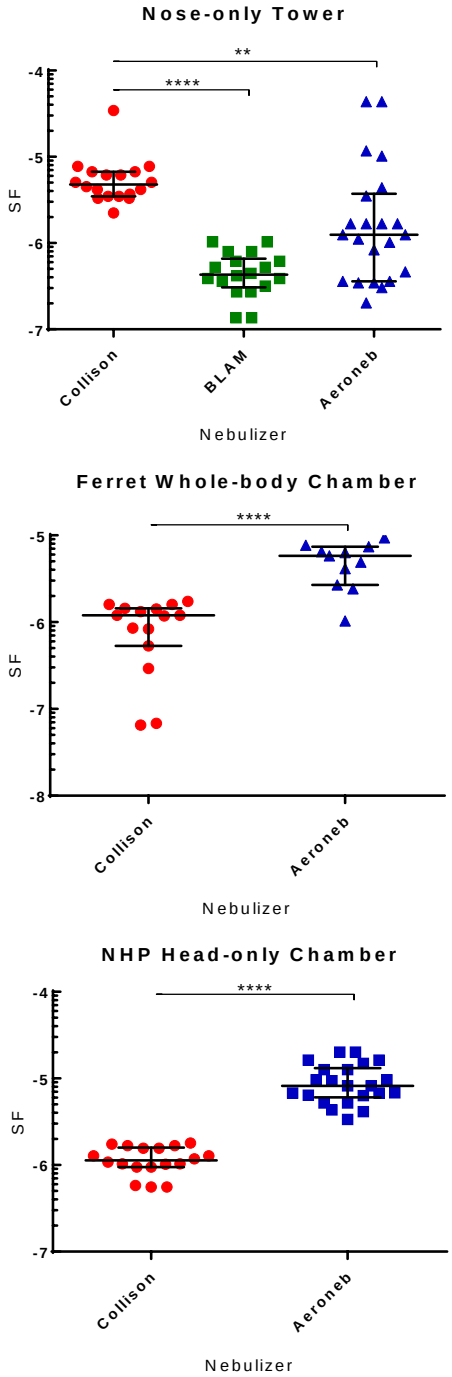

Figure 7. Spray Factor of Influenza is Higher with the Collison in the NOT and Higher with the Aeroneb in the FWB and NHP HO Chambers

The Collison is represented by red symbols, the BLAM by green symbols, and the Aeroneb by blue symbols. Error bars represent the median and interquartile range. In the NOT, the SF is significantly different between the Collison and the BLAM (p-value<0.0001) and the Collison and the Aeroneb (p-value=0.0145). The SF was significantly different between the Collison and the Aeroneb in the FWB chamber (p-value<0.0001) and the NHP head-only (p-value<0.0001).

Addition of fluorescein salt to influenza samples was assessed prior to aerosol experiments to insure fluorescein did not neutralize influenza or interfere with influenza infection and lysis of MDCK cells. Tenfold serial dilutions of fluorescein salt in ddH2O were made from 1mg/ml to 0.0001mg/ml. Five virus samples were set up with 3.45ml of VGM, 500µl of influenza virus, and 50µl of fluorescein salt. A control sample was set up with 4.5ml VGM and 500µl of influenza virus. Samples were incubated on ice for one hour to simulate aerosol conditions. Three tenfold serial dilutions of each concentration of fluorescein sample were prepared in 1X PBS to simulate loss during aerosolization, which for influenza is about a two log difference between the nebulizer and the AGI. Of the five fluorescein concentrations only three fluorescein concentrations were able to be read at all three dilutions: 1.0mg/ml, 0.1mg/ml, and 0.01mg/ml. Titer of these samples was determined by plaque assays to assess changes in recovery of live virus. Table 7 shows the results of the plaque assays for each fluorescein concentration. All titers were within typical variation in the plaque assay method. The 1.0mg/ml fluorescein salt sample had the highest change in titer from the control (+0.79 PFU/ml). The 0.1mg/ml fluorescein salt resulted in 0.48 PFU/ml increase in titer, while the 0.01mg/ml fluorescein salt resulted in a 0.31 PFU/ml decrease in titer. For the fluorescein salt aerosols, a concentration of 0.1mg/ml of fluorescein salt was selected due to its minimal interference in concentration and detectability at several dilutions lower.

Table 7 Effect of Fluorescein Salt on Influenza A Titer

Sample	Influenza Titer
Control	2.95E05 PFU/ml
1.0mg/ml fluorescein	3.74E05 PFU/ml
0.1mg/ml fluorescein	3.43E05 PFU/ml
0.01mg/ml fluorescein	2.64E05 PFU/ml

Table 8 summarizes the SF results for fluorescein and influenza. In the NOT, the Collison resulted in a -0.10 log reduction in SF, the BLAM resulted in a 0.78 log reduction in SF, and the Aeroneb resulted in a 0.36 log reduction in SF. In the FWB chamber, the Collison resulted in a 0.72 log reduction in SF, while the Aeroneb resulted in a 0.06 log reduction in SF. In the NHP HO chamber, the Collison resulted in a 0.65 log reduction in SF and the Aeroneb resulted in a 0.12 log reduction in SF. Figure 8 shows a graphical representation for SF results for fluorescein salt and influenza in each exposure chamber. There was a significant difference in SF between fluorescein and influenza with the BLAM in the NOT (p-value<0.0001) and the Collison in both the FWB chamber and NHP HO chamber (p-value<0.0001 for both).

Table 8 Log Reduction in SF for Influenza in Different Exposure Chambers

NOT			
Aerosol Generator	Median Fluorescein SF	Median Influenza SF	Log Reduction
Collison	3.44E-06	4.34E-06	-0.10
BLAM	2.59E-06	4.32E-07	0.78
Aeroneb	2.68E-06	1.18E-06	0.36
FWB			
Collison	6.35E-06	1.20E-06	0.72
Aeroneb	6.73E-06	5.80E-06	0.06
NHP HO			
Collison	5.07E-06	1.13E-06	0.65
Aeroneb	9.97E-06	7.52E-06	0.12

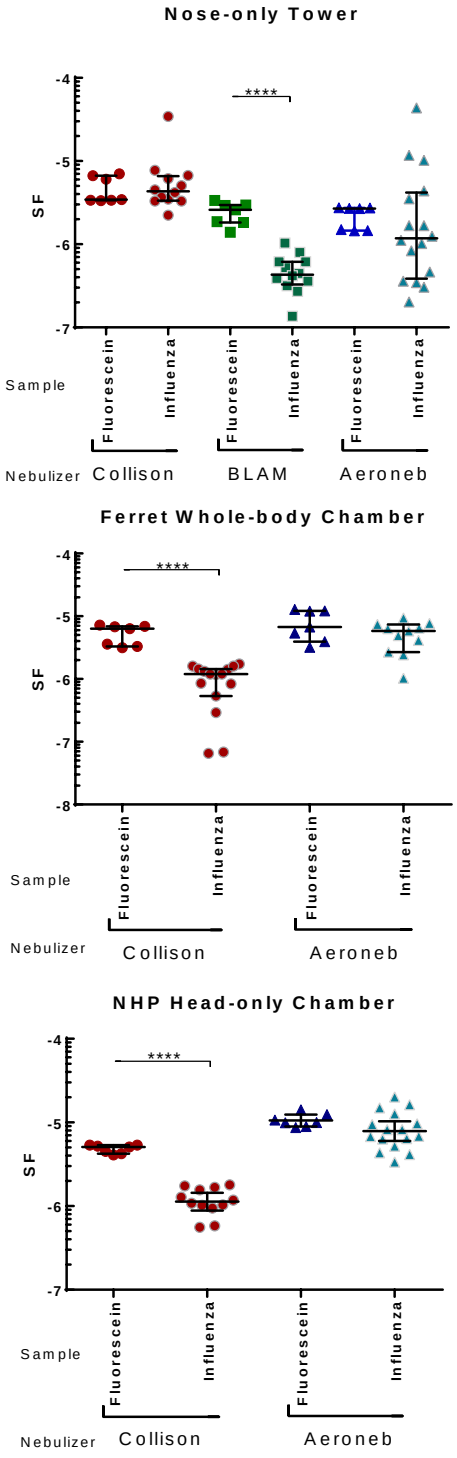

Figure 8. Significant Decrease in Influenza Viability with the BLAM in the NOT and the Collison in the FWB and NHP HO Chambers

Red symbols represent the Collison, green symbols the BLAM, and blue symbols the Aeroneb. Error bars represent the median and interquartile range. Fluorescein salt SF represents the maximum achievable SF for each aerosol generator, while influenza SF represents the actual SF achieved using a live virus.

Median aerosol efficiency is summarized in Table 9 for each aerosol generator. In the NOT, aerosol efficiency is highest for the Collison (13.87%), followed by the BLAM (5.41%) and the Aeroneb (2.10%). Both the BLAM and the Aeroneb are significantly different from the Collison (p-value=0.0004 and p-value=0.0021, respectively. In the FWB chamber, the aerosol efficiency is higher for the Aeroneb (31.91%) than the Collison (6.84%). The aerosol efficiency of the Aeroneb (32.27%) is higher than the Collison (5.35%) in the NHP HO chamber. The aerosol efficiency of the Aeroneb is significantly different from the Collison in both the FWB (p-value<0.0001) and NHP HO chambers (p-value<0.0001).

Table 9 Aerosol Efficiency of Influenza in Different Exposure Chambers

NOT		
Aerosol Generator	Median Aerosol Efficiency	P-value
Collison	13.87%	
BLAM	5.41%	**0.0004**
Aeroneb	2.10%	**0.0021**
FWB		
Collsion	6.84%	
Aeroneb	31.91%	**<0.0001**
NHP HO		
Collison	5.35%	
Aeroneb	32.27%	**<0.0001**
*Based on a two-sided Mann-Whitney U test **Signficant results in bold		

Particle size data was only collected for H3N2 with the Collison in the NOT and for H5N1 with the Aeroneb in the NHP HO chamber (Figure 9). In the NOT, the average count median aerodynamic diameter (CMAD) was 1.41µm and the average mass median aerodynamic diameter (MMAD) was 4.16µm for the Collison. In the NHP HO chamber, the Aeroneb produced particles with an average CMAD of 1.46µm and an average MMAD of 3.41µm. Issues with the particle sizer prevented a more rigorous assessment of particle size with these studies.

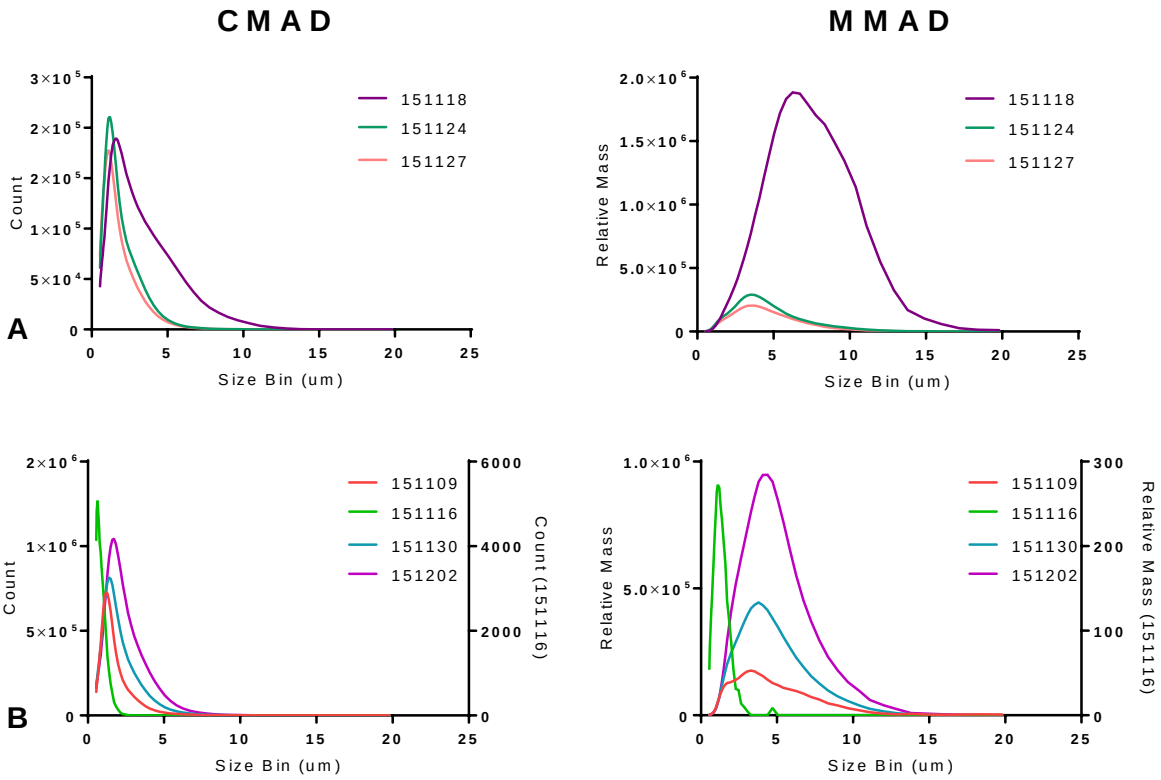

Figure 9. The Collison Produces Smaller Aerosol Particles than the Aeroneb
The CMAD and MMAD for influenza in the A) NOT with the Collison and B) in the NHP HO chamber with the Aeroneb. For the Collison, the CMAD was 1.41µm and MMAD was 4.16µm. In the NHP HO chamber, the Aeroneb produced particles with a CMAD of 1.46µm and an MMAD of 3.41µm.

4.1.3 Rift Valley Fever Virus

Aerosolization of Rift Valley Fever Virus (RVFV) was assessed in the rodent whole-body (RWB) chamber for the Collison and the Aeroneb. Spray factor (SF) results for RVFV are show in Figure 9 and summarized in Table 10. Plaque assays from Aeroneb aerosols with RVFV were done by Amy Hartman and Michael Kujawa. Collison data was based on previous aerosol studies performed in 2010 by Doug Reed, Le'Kneitah Smith, Amy Hartman and Laura Bethel since current studies supplement with humidity, potentially altering the SF. The average SF of RVFV for the Aeroneb (1.26E-06) was higher than the Collison (3.63E-07). The CV was much higher for the Collison (150.66%) than the Aeroneb (59.87%). The SF of RVFV in the Aeroneb was significantly different from the Collison based on a two-sided Mann-Whitney U test (p-value=0.0073).

Table 10 Summary of SF Results for RVFV Aerosols

	Collison	Aeroneb
Mean	3.63E-07	1.26E-06
SD	5.47E-07	7.56E-07
CV	150.66%	59.87%

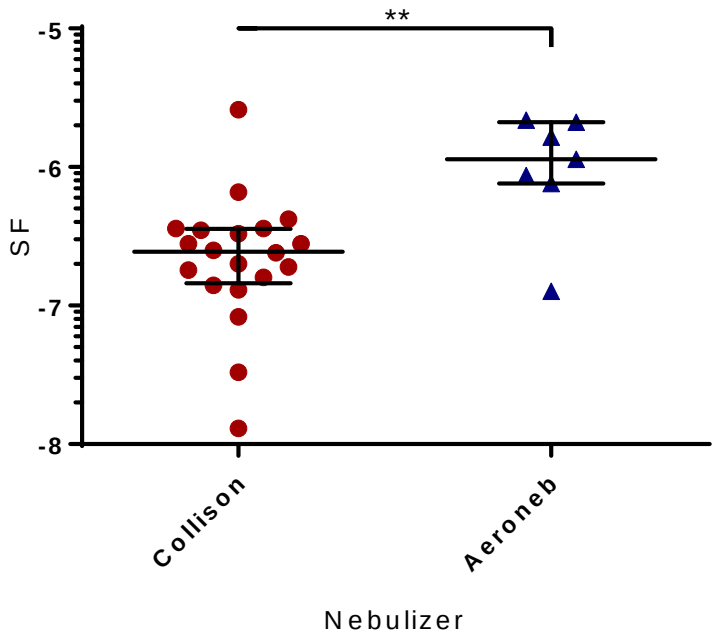

Figure 10. Spray Factor of RVFV is Higher with the Aeroneb in the RWB Chamber
The Collison is represented by red symbols and the Aeroneb is represented by blue symbols. Error bars represent the median and interquartile range. Using a two-sided Mann-Whitney U test, the difference in spray factor between the Collison and the Aeroneb is significant (p-value=0.0073).

Table 11 summarizes the aerosol efficiency of the Collison and the Aeroneb for RFVF. Collison data was taken from aerosols performed in 2010; aerosols were done by Dr. Doug Reed and Le'Kneitah Smith while plaque assays were done by Amy Hartman and Laura Bethel. During this time, amount of sample nebulized was not recorded, and thus an assumed 3mls of volume aerosolized was used for calculation of aerosol efficiency for the Collison based on the average amount nebulized by the Collison for LVS and influenza in these studies. The aerosol efficiency of the Aeroneb (6.38%) was higher than the Collison (1.59%). The difference in SF is significant based on a two-sided Mann-Whitney U test (p-value=0.0037).

Table 11 Aerosol Efficiency of RVFV in the RWB Chamber

Aerosol Generator	Median Aerosol Efficiency	P-value*
Collison	1.59%	
Aeroneb	6.38%	**0.0037**
*Based on a two-sided Mann-Whitney U test **signficant results in bold		

Particle size for RVFV aerosols was only collected for the Aeroneb due to issues with the particle sizer, shown in Figure 11. In the RWB chamber, the Aeroneb produced particles with an average CMAD of 1.42µm and an average MMAD of 3.34µm.

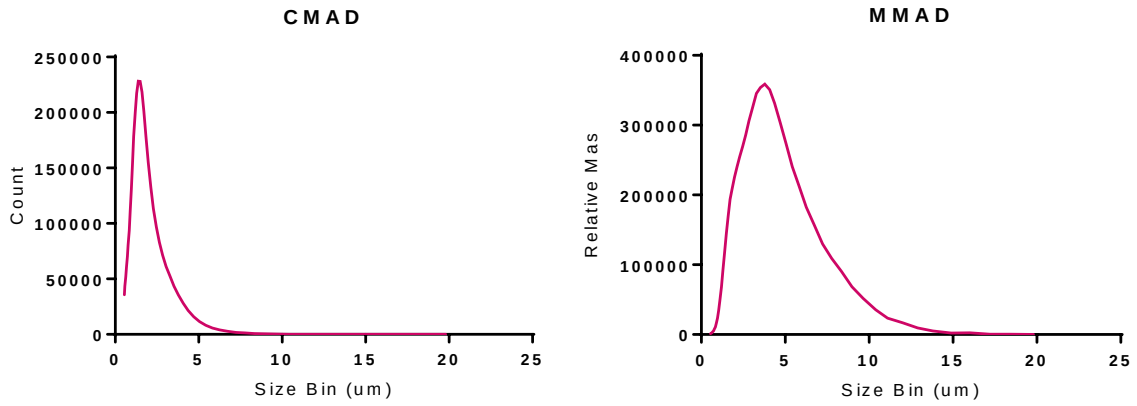

Figure 11. CMAD and MMAD for RVFV Aerosols

In the RWB chamber, the Aeroneb produced RVFV particles with an average CMAD of 1.42µm and an average MMAD of 3.34µm.

4.2 ENVIRONMENTAL PARAMETERS

The AeroMP system controls the aerosol airflow parameters and records environmental data, such as relative humidity, temperature and sampler pressure within the exposure system. Due to the differing methodologies employed by the aerosol generators to produce aerosols, environmental factors may vary between aerosol generators.

4.2.1 *Francisella tularensis*

Tables 12 and 13 summarize the relative humidity (RH) and temperature, respectively, achieved by each aerosol generator during LVS aerosols. RH in the NOT ranged from 62.29% to 101.66% with a median of 86.63% for the Collison. The RH in the NOT for the BLAM ranged slightly lower (48.94% to 94.44%) with a median of 75.25%. The RH for the Aeroneb ranged from 54.48% to 101.67% with a median of 67.20% in the NOT. In the RWB chamber, the RH for the Collison ranged from 39.56% to 77.42% with a median of 55.25%. The RH with the Aeroneb in the RWB chamber ranged from 46.09% to 90.47% with a median RH of 74.63%. The median temperature in the NOT was with the Collison (22.22°C), followed by the Aeroneb (21.94°C) and BLAM (21.86°C). The median temperature was 21.79°C with the Collison and 21.71°C with the Aeroneb in the RWB chamber.

Table 12 Summary of RH During LVS Aerosols

	NOT			RWB	
	Collison	BLAM	Aeroneb	Collison	Aeroneb
Range	62.29% 101.66%	48.94% 94.44%	54.48% 101.67%	39.56% 77.42%	46.09% 90.47%
Median	86.63%	75.25%	67.21%	55.25%	74.63%

Table 13 Summary of Temperature During LVS Aerosols

	NOT			RWB	
	Collison	BLAM	Aeroneb	Collison	Aeroneb
Range	20.67°C 23.15°C	20.93°C 22.79°C	20.46°C 23.64°C	21.21°C 23.12°C	21.11°C 22.98°C
Median	22.22°C	21.86°C	21.94°C	21.79°C	21.71°C

Multiple linear regression analysis was performed to predict SF from RH, temperature, and sampler pressure. Results of the regression summarized in Tables 14-18. These variables significantly predicted SF in the NOT for the Collison (p-value=0.0302), but not for the BLAM (p-value= 0.1177) or the Aeroneb (p-value=0.5435). Of the four variables tested, only the RH (p-value=0.006) added statistically significantly to the prediction. In the RWB chamber, multiple linear regression analysis was statistically significant for the Aeroneb (p-value<0.0001) but not the Collison (p-value=0.3640). All parameters contributed statistical significance to the prediction for the Aeroneb in the RWB chamber.

Table 14 Linear Regression of LVS with the Collison in the NOT

Source	SS	df	MS	Number of obs	=	16
				F(3, 12)	=	4.19
Model	1.2059e-12	3	4.0197e-13	Prob > F	=	0.0302
Residual	1.1503e-12	12	9.5857e-14	R-squared	=	0.5118
				Adj R-squared	=	0.3898
Total	2.3562e-12	15	1.5708e-13	Root MSE	=	3.1e-07

SF	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
RH	-1.84e-08	5.47e-09	-3.37	0.006	-3.03e-08	-6.50e-09
Temp	-8.00e-08	1.33e-07	-0.60	0.558	-3.70e-07	2.10e-07
SampPress	6.09e-08	2.83e-08	2.15	0.053	-8.93e-10	1.23e-07
_cons	4.47e-06	3.28e-06	1.36	0.198	-2.68e-06	.0000116

Table 15 Linear Regression of LVS with the BLAM in the NOT

Source	SS	df	MS	Number of obs	=	11
				F(3, 7)	=	2.81
Model	4.4971e-14	3	1.4990e-14	Prob > F	=	0.1177
Residual	3.7360e-14	7	5.3372e-15	R-squared	=	0.5462
				Adj R-squared	=	0.3517
Total	8.2331e-14	10	8.2331e-15	Root MSE	=	7.3e-08

SF	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
RH	6.84e-09	4.50e-09	1.52	0.172	-3.80e-09	1.75e-08
Temp	7.99e-08	5.51e-08	1.45	0.190	-5.03e-08	2.10e-07
SampPress	-9.54e-09	1.47e-08	-0.65	0.536	-4.42e-08	2.51e-08
_cons	-2.20e-06	1.58e-06	-1.39	0.206	-5.93e-06	1.53e-06

Table 16 Linear Regression of LVS with the Aeroneb in the NOT

Source	SS	df	MS	Number of obs	=	7
				F(3, 3)	=	0.87
Model	2.2019e-17	3	7.3396e-18	Prob > F	=	0.5435
Residual	2.5248e-17	3	8.4158e-18	R-squared	=	0.4658
				Adj R-squared	=	-0.0683
Total	4.7266e-17	6	7.8777e-18	Root MSE	=	2.9e-09

SF	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
RH	-4.70e-10	4.55e-10	-1.03	0.377	-1.92e-09	9.78e-10
Temp	-4.25e-09	4.51e-09	-0.94	0.416	-1.86e-08	1.01e-08
SampPress	1.39e-09	1.45e-09	0.96	0.407	-3.21e-09	5.99e-09
_cons	1.53e-07	1.00e-07	1.53	0.224	-1.66e-07	4.72e-07

Table 17 Linear Regression of LVS with the Collison in the RWB Chamber

Source	SS	df	MS	Number of obs	=	15
				F(3, 11)	=	1.17
Model	3.0044e-17	3	1.0015e-17	Prob > F	=	0.3640
Residual	9.3870e-17	11	8.5337e-18	R-squared	=	0.2425
				Adj R-squared	=	0.0359
Total	1.2391e-16	14	8.8510e-18	Root MSE	=	2.9e-09

SF	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
RH	3.92e-11	8.77e-11	0.45	0.664	-1.54e-10	2.32e-10
Temp	-4.92e-09	3.23e-09	-1.52	0.157	-1.20e-08	2.20e-09
SampPress	-1.30e-10	2.75e-10	-0.47	0.647	-7.36e-10	4.76e-10
_cons	1.06e-07	7.15e-08	1.48	0.168	-5.18e-08	2.63e-07

Table 18 Linear Regression of LVS with the Aeroneb in the RWB Chamber

Source	SS	df	MS	Number of obs	=	15
Model	6.4685e-14	3	2.1562e-14	F(3, 11)	=	29.53
Residual	8.0311e-15	11	7.3010e-16	Prob > F	=	0.0000
Total	7.2717e-14	14	5.1940e-15	R-squared	=	0.8896
				Adj R-squared	=	0.8594
				Root MSE	=	2.7e-08

SF	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
RH	5.92e-09	1.53e-09	3.88	0.003	2.56e-09	9.28e-09
Temp	1.59e-07	2.11e-08	7.51	0.000	1.12e-07	2.05e-07
SampPress	-2.06e-08	5.52e-09	-3.74	0.003	-3.28e-08	-8.50e-09
_cons	-4.00e-06	5.17e-07	-7.74	0.000	-5.14e-06	-2.86e-06

4.2.2 Influenza

The RH and temperature achieved for each aerosol generator is summarized in Table 14 and Table 15. In the NOT, the RH ranged between 69.02% and 101.64% with a median of 82.55% for the Collison, 53.29% to 96.66% with a median of 80.46% for the BLAM, and 58.59% to 101.66% with a median of 92.22% for the Aeroneb. In the FWB chamber, the RH ranged from 39.23% to 97.67% with a median of 66.25% for the Collison and 47.52% to 88.08% with a median of 62.11% for the Aeroneb. The RH ranged from 73.01% to 92.51% with a median of 80.96% for the Collison and 86.05% to 101.67% with a median of 101.63% for the Aeroneb in the NHP HO chamber. The median temperature was highest for the Aeroneb (22.01°C), followed by the Collison (21.83°C) and the BLAM (21.50°C) in the NOT. In the FWB, the median temperature was 22.26°C for the Collison and 22.44°C for the Aeroneb. The median

temperature for the Collison was 21.51°C for the Collison and 21.35°C for the Aeroneb in the NHP HO chamber.

Table 19 Summary of RH During Influenza Aerosols

	NOT			FWB		NHP HO	
	Collison	BLAM	Aeroneb	Collison	Aeroneb	Collison	Aeroneb
Range	69.02% 101.64%	53.29% 96.66%	58.59% 101.66%	39.23% 97.67%	47.52% 88.08%	73.01% 92.51%	86.05% 101.67%
Median	82.54%	80.46%	92.21%	66.25%	62.11%	80.95%	101.62%

Table 20 Summary of Temperature During Influenza Aerosols

	NOT			FWB		NHP HO	
	Collison	BLAM	Aeroneb	Collison	Aeroneb	Collison	Aeroneb
Range	20.76°C 23.41°C	20.71°C 23.40°C	20.92°C 23.56°C	20.80°C 26.81°C	20.55°C 23.26°C	20.62°C 22.49°C	19.57°C 23.77°C
Median	21.83°C	21.50°C	22.01°C	22.26°C	22.44°C	21.51°C	21.35°C

Multiple linear regression to predict SF from RH, temperature, and sampler pressure was statistically significant for the BLAM (p-value<0.0001) in the NOT, the Aeroneb (p-value=0.0045) in the FWB chamber, and the Collison (p-value=0.0054) in the NHP HO chamber. For the BLAM in the NOT temperature (p-value=0.011) and sampler pressure (p-value=0.049) contributed significantly to prediction, while only sampler pressure contributed significantly to the prediction for the Aeroneb in the FWB chamber (p-value=0.004). Only RH contributed statistically significantly to the prediction for the Collison in the NHP HO chamber (p-value=0.001).

Table 21 Linear Regression of Influenza with the Collison in the NOT

Source	SS	df	MS	Number of obs	=	14
				F(3, 10)	=	2.10
Model	3.1748e-09	3	1.0583e-09	Prob > F	=	0.1636
Residual	5.0348e-09	10	5.0348e-10	R-squared	=	0.3867
				Adj R-squared	=	0.2027
Total	8.2096e-09	13	6.3151e-10	Root MSE	=	2.2e-05

SF	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
RH	2.92e-07	7.71e-07	0.38	0.713	-1.43e-06	2.01e-06
Temp	5.52e-06	9.77e-06	0.56	0.585	-.0000163	.0000273
SampPress	-6.99e-06	5.28e-06	-1.32	0.215	-.0000188	4.77e-06
_cons	-.0001905	.0002572	-0.74	0.476	-.0007635	.0003825

Table 22 Linear Regression of Influenza with the BLAM in the NOT

Source	SS	df	MS	Number of obs	=	19
				F(3, 15)	=	19.86
Model	6.8215e-12	3	2.2738e-12	Prob > F	=	0.0000
Residual	1.7174e-12	15	1.1449e-13	R-squared	=	0.7989
				Adj R-squared	=	0.7587
Total	8.5388e-12	18	4.7438e-13	Root MSE	=	3.4e-07

SF	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
RH	-1.45e-08	9.32e-09	-1.55	0.141	-3.44e-08	5.38e-09
Temp	-4.70e-07	1.62e-07	-2.91	0.011	-8.14e-07	-1.26e-07
SampPress	-9.35e-08	3.87e-08	-2.42	0.029	-1.76e-07	-1.10e-08
_cons	.0000114	4.34e-06	2.62	0.019	2.13e-06	.0000206

Table 23 Linear Regression of Influenza with the Aeroneb in the NOT

Source	SS	df	MS	Number of obs	=	23
				F(3, 19)	=	2.35
Model	4.8863e-10	3	1.6288e-10	Prob > F	=	0.1052
Residual	1.3193e-09	19	6.9439e-11	R-squared	=	0.2703
				Adj R-squared	=	0.1550
Total	1.8080e-09	22	8.2180e-11	Root MSE	=	8.3e-06

SF	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
RH	-3.94e-07	1.50e-07	-2.63	0.016	-7.07e-07	-8.10e-08
Temp	-1.95e-06	2.59e-06	-0.75	0.461	-7.37e-06	3.47e-06
SampPress	5.47e-07	4.84e-07	1.13	0.273	-4.66e-07	1.56e-06
_cons	.0000865	.0000652	1.33	0.200	-.0000499	.0002229

Table 24 Linear Regression of Influenza with the Collison in the FWB Chamber

Source	SS	df	MS	Number of obs	=	15
				F(3, 11)	=	2.29
Model	1.6686e-12	3	5.5619e-13	Prob > F	=	0.1350
Residual	2.6718e-12	11	2.4289e-13	R-squared	=	0.3844
				Adj R-squared	=	0.2165
Total	4.3403e-12	14	3.1002e-13	Root MSE	=	4.9e-07

SF	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
RH	-1.55e-08	9.61e-09	-1.61	0.135	-3.67e-08	5.65e-09
Temp	8.41e-08	1.15e-07	0.73	0.480	-1.69e-07	3.37e-07
SampPress	-9.09e-08	2.14e-07	-0.42	0.679	-5.62e-07	3.80e-07
_cons	-5.98e-07	2.32e-06	-0.26	0.802	-5.72e-06	4.52e-06

Table 25 Linear Regression of Influenza with the Aeroneb in the FWB Chamber

Source	SS	df	MS	Number of obs	=	18
				F(3, 14)	=	6.87
Model	9.0013e-11	3	3.0004e-11	Prob > F	=	0.0045
Residual	6.1146e-11	14	4.3675e-12	R-squared	=	0.5955
				Adj R-squared	=	0.5088
Total	1.5116e-10	17	8.8917e-12	Root MSE	=	2.1e-06

SF	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
RH	-4.89e-08	1.19e-07	-0.41	0.688	-3.04e-07	2.07e-07
Temp	1.40e-06	1.68e-06	0.83	0.420	-2.21e-06	5.01e-06
SampPress	-8.94e-07	2.61e-07	-3.43	0.004	-1.45e-06	-3.35e-07
_cons	-.0000291	.0000445	-0.65	0.524	-.0001245	.0000663

Table 26 Linear Regression of Influenza with the Collison in the NHP HO Chamber

Source	SS	df	MS	Number of obs	=	12
				F(3, 8)	=	9.33
Model	1.5028e-12	3	5.0094e-13	Prob > F	=	0.0054
Residual	4.2944e-13	8	5.3680e-14	R-squared	=	0.7778
				Adj R-squared	=	0.6944
Total	1.9322e-12	11	1.7566e-13	Root MSE	=	2.3e-07

SF	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
RH	-6.73e-08	1.36e-08	-4.96	0.001	-9.85e-08	-3.60e-08
Temp	-6.92e-08	1.71e-07	-0.41	0.696	-4.63e-07	3.24e-07
SampPress	7.14e-08	6.00e-08	1.19	0.269	-6.70e-08	2.10e-07
_cons	8.55e-06	4.27e-06	2.00	0.080	-1.29e-06	.0000184

Table 27 Linear Regression of Influenza with the Aeroneb in the NHP HO Chamber

Source	SS	df	MS	Number of obs	=	23
Model	7.1099e-11	3	2.3700e-11	F(3, 19)	=	1.48
Residual	3.0412e-10	19	1.6007e-11	Prob > F	=	0.2516
Total	3.7522e-10	22	1.7056e-11	R-squared	=	0.1895
				Adj R-squared	=	0.0615
				Root MSE	=	4.0e-06

SF	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
RH	-3.73e-07	1.93e-07	-1.94	0.068	-7.76e-07	3.03e-08
Temp	-7.72e-07	7.35e-07	-1.05	0.307	-2.31e-06	7.66e-07
SampPress	-4.11e-07	8.51e-07	-0.48	0.634	-2.19e-06	1.37e-06
_cons	.0000588	.0000305	1.93	0.069	-4.99e-06	.0001225

4.2.3 Rift Valley Fever Virus

Table 16 and Table 17 summarize the RH and temperature achieved by each aerosol generator during Rift Valley Fever Virus (RVFV) aerosols in the Rodent whole-body chamber (RWB). The median RH achieved by the Collison was 38.41% with a range of 25.68% to 46.03%. The RH with the Aeroneb ranged from 79.86% to 89.14% with a median RH of 83.40%. The median temperature was 23.36°C with the Collison and 22.77°C with the Aeroneb.

Table 28 Summary of RH During RVFV Aerosols

	Collison	Aeroneb
Range	25.68% 46.03%	79.86% 89.14%
Median	38.41%	83.40%

Table 29 Summary of Temperature During RVFV Aerosols

	Collison	Aeroneb
Range	20.64°C 25.69°C	22.27°C 23.81°C
Median	23.36°C	22.77°C

Multiple linear regression analysis to predict SF from RH, temperature, sampler pressure, and outside relative humidity was not statistically significant for either the Collison or the Aeroneb in the RWB chamber.

Table 30 Linear Regression of RVFV with the Collison in the RWB Chamber

```
      Source |       SS           df       MS      Number of obs   =        20
-------------+----------------------------------   F(2, 17)        =      1.03
       Model |  5.9585e-13         2  2.9792e-13   Prob > F        =    0.3778
    Residual |  4.9108e-12        17  2.8887e-13   R-squared       =    0.1082
-------------+----------------------------------   Adj R-squared   =    0.0033
       Total |  5.5066e-12        19  2.8982e-13   Root MSE        =    5.4e-07
------------------------------------------------------------------------------
          SF |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          RH |   1.12e-08   3.90e-08     0.29   0.777    -7.10e-08    9.34e-08
        Temp |   3.95e-07   4.07e-07     0.97   0.346    -4.64e-07    1.25e-06
       _cons |  -9.16e-06   .0000106    -0.86   0.402    -.0000316    .0000133
       _cons |   .0000588   .0000305     1.93   0.069    -4.99e-06    .0001225
```

Table 31 Linear Regression of RVFV with the Aeroneb in the RWB Chamber

Source	SS	df	MS	Number of obs	=	7
				F(3, 3)	=	6.99
Model	2.9959e-12	3	9.9863e-13	Prob > F	=	0.0722
Residual	4.2841e-13	3	1.4280e-13	R-squared	=	0.8749
				Adj R-squared	=	0.7498
Total	3.4243e-12	6	5.7072e-13	Root MSE	=	3.8e-07

SF	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
RH	-2.22e-07	1.21e-07	-1.84	0.163	-6.06e-07	1.62e-07
Temp	-3.01e-07	6.16e-07	-0.49	0.659	-2.26e-06	1.66e-06
SampPress	3.71e-09	1.99e-07	0.02	0.986	-6.28e-07	6.36e-07
_cons	.000027	6.62e-06	4.08	0.027	5.95e-06	.0000481

5.0 DISCUSSION

Aerobiology is the study of airborne biological particulates, such as bacteria, viruses, or pollen, and the factors that affect the dispersion of these airborne particles. Aerosol research is necessary for studying infections spread through the respiratory route and developing therapeutics and vaccines. Prior to beginning studies with animal models it is important to characterize and understand the effect different aerosol techniques, equipment, and environmental parameters can have on the reliability and reproducibility of a research design.

Aerosol performance can be affected by a variety of different factors. Pre-aerosolization factors occur prior to the aerosol experiment and include pathogen growth conditions or equipment selection [53]. Faith et al. showed aerosol performance of LVS improved when the bacterium was grown in BHI broth rather than Mueller Hinton Broth; [24]. other studies suggest bacteria are more resistant to the effects of aerosolization in the resting or lag phase of growth [53]. Aerosolization factors are factors that occur during the actual aerosol experiment, and can include relative humidity, temperature, or air leaks. Post-aerosolization factors describe events that occur after the aerosol and are usually dependent on host factors of the animal model, such as susceptibility [53].

This study only examined a few factors that can potentially affect aerosol performance, but attempts were made to control potential confounding factors. The same method was used to culture bacteria and the same stock of virus was used for aerosols. While RH and temperature

were not controlled and others were hard to predict (such as air leaks), the parameters of the aerosol system were kept constant for each pathogen. Animals were not used in these studies to avoid the potential for bias based on disease outcome of the animal models.

Aerosol performance of the BLAM and the Aeroneb was statistically different from the Collison for LVS, influenza, and RVFV in all exposure chambers tested. In the NOT, the Collison achieved a higher SF and aerosol efficiency than both the BLAM and the Aeroneb. However, in the RWB, FWB, and NHP HO chambers, the Aeroneb achieved a higher SF and aerosol efficiency than the Collison for LVS, influenza, and RVFV. While the Aeroneb showed data suggesting better aerosol performance with certain exposure chambers, the BLAM did not exceed the aerosol performance of the Collison for any of the organisms.

Interestingly, influenza spray factor was not significantly different between H1N1 and H3N2 with the Collison in the FWB or the NHP HO chamber, but H3N2 had a significantly different spray factor from H1N1 and H5N1 in the NHP HO chamber with the Aeroneb. The data with the Aeroneb correlates with previous studies indicating H3N2 has an improved transmissibility compared to H1N1[54]. However, it is unclear why this trend is not seen between the two strains with the Collison. This data suggests that H1N1 can be used as a surrogate for H5N1 when testing and selecting aerosol equipment for aerosol experiments.

It is important to note the Collison and the BLAM used 10mls of sample for aerosol generation while the Aeroneb only used 5mls. In a ten-minute aerosol, the Collison aerosolized an average of 3mls of sample, the BLAM approximately 1ml of sample, and the Aeroneb an average of 4mls of sample. While the SF allows for determination of starting concentration needed to reach a target dose, the SF does not account for potential increases in aerosol performance due to volume of sample aerosolized. Aerosol performance was also assessed by

aerosol efficiency, which is the ratio of the actual aerosol concentration to the theoretical aerosol concentration as determined by the volume and concentration aerosolized. For each aerosol generator, aerosol efficiency correlated with the SF results. This indicates that the improvement in SF for the Aeroneb compared to the Collison in the whole-body and head-only chambers are not due to rate at which liquid is aerosolized.

Prior studies suggest the Collison may cause damage to pathogens during aerosolization due to the recirculation of sample. Fluorescein salt was added to some experiments to act as a surrogate for the microorganisms to determine the ideal SF of each aerosol generator. The small size and lack of a membrane ensure the fluorescein salt will not be damaged during aerosolization, and thus any loss of sample can be attributed to leaks in the exposure system or adhesion of aerosol particles to equipment, such as the walls of the exposure chamber. Aerosol performance for pathogens can be affected by the natural loss due to the exposure system and loss of viability of the organism during aerosolization. A lower SF for the pathogen relative to that for fluorescein salt is assumed to be due to loss of viability of the pathogen. Loss of organism viability correlated with SF and aerosol efficiency results. In the NOT, there was less reduction in SF for LVS and influenza relative to fluorescein with the Collison, while in the whole-body and head-only chambers there was less of a reduction in SF relative to fluorescein with the Aeroneb for LVS, influenza, and RVFV.

Despite the modified configuration of the BLAM and the use of single pass atomization, the Collison still had better aerosol performance as measured by the SF, aerosol efficiency, and loss of viability. Interestingly, the Aeroneb had better aerosol performance than the Collison for all organisms in the whole-body and head-only chambers, but not in the NOT. A potential reason for this discrepancy in performance could be due to the length of the mixing tube. The

whole-body and head-only chambers have a longer mixing tube than the NOT. Thus, in the NOT aerosol particles do not have as much time to desiccate. Particles may remain larger which could improve survival. The AeroMP was programmed to use 0.5 L/min of dilution air with the Collison while the BLAM needed no dilution air. The shorted mixing tube of the NOT can result in larger aerosol particles that can condense out of the aerosol. The dilution air aids in desiccation of aerosol particles. The lack of dilution air used with the BLAM could explain the poor aerosol performance of the BLAM compared to the Collison. The poor performance of the Aeroneb with the NOT was surprising. Unlike the Collison and BLAM which used the short mixing tube of the NOT to dry out aerosol particles, the Aeroneb used tubing to connect to the mixing tube of the NOT. For the Aeroneb, the length of tubing from the generator to the NOT may affect desiccation of aerosol particles, with longer tubing resulting in too much desiccation, and shorter tubing resulting in inadequate desiccation. In agreement with this, the reduction in SF for LVS with the Aeroneb in the NOT relative to fluorescein was the highest of any of the nebulizer/chamber/pathogen combinations tested. Length of tubing from the aerosol generator to the NOT was not examined in this study and thus is a potential factor to examine to determine if there is an effect on aerosol performance in the NOT.

To evaluate whether other aspects of the aerosol setup impact aerosol performance, a multiple linear regression was performed for each aerosol set up to determine if RH, temperature, and sampler pressure affect SF. RH contributed significantly to the prediction in both the NOT and the RWB chamber, but RH had an inverse relationship with the SF in the NOT, meaning the SF increases as RH decreases in this exposure chamber. This could be due to the shorter mixing tube. A higher humidity could reduce desiccation, potentially causing aerosol particles to condense out of the aerosol. RH and temperature were positive predictors for the SF of LVS

with the Aeroneb in the RWB chamber. This data correlates with previous studies indicating humidity lower the 55% reduces the SF [24]. While the relationship was not seen with the Collison in the RWB chamber, the median RH of the Collison suggests the RH was often low enough to reduce aerosol performance.

The multiple regression significantly predicted the SF of influenza with the BLAM in the NOT, the Aeroneb in the FWB chamber, and the Collison in the NHP HO chamber. For the BLAM in the NOT, temperature and sampler pressure contributed significantly to the prediction model. For the Aeroneb in the FWB chamber, sampler pressure is statistically significant to the model. In the NHP HO chamber with the Collison, RH contributed significantly to the model. While the model is not significant for the other setups, RH is significantly correlated with SF for the Aeroneb in the NOT and results are suggestive that RH is correlated with the Aeroneb in the NHP HO chamber. In all cases, the RH was a negative predictor for the SF, indicating improved aerosol performance of influenza may be correlated with lower humidity. This is intriguing because epidemiological studies and transmission studies in animal models have suggested that influenza transmissibility is higher at lower humidity. Why lower humidity would correspond with increased transmission is not clear.

In the RWB chamber with the Aeroneb, there was a positive correlation between temperature and LVS SF. While the range of temperature with the Collison and Aeroneb was very narrow, the Aeroneb had a slightly lower median temperature. This suggests that similar to RH, there is a point at which temperature affects aerosol performance of LVS. In the NOT with the BLAM, temperature was a negative predictor for the SF of influenza. Thus, this data suggests influenza has improved survival in lower temperatures. However, due to the lack of correlation between temperature and SF with the other aerosol generators and the small range of

temperature achieved during the aerosols these findings could be a spurious correlation. Sample pressure was an inverse predictor for the BLAM and the Aeroneb. In the aerosol exposure system, the sampler pressure is typically less than -6.0in Hg. Aerosol performance of several aerosols was much lower than expected, leading to an investigation of the cause. Examination of the recorded aerosol parameters revealed higher sampler pressures are associated with poor aerosol performance. Thus, the regression models showing an inverse relationship between SF and sampler pressure supports this observation. Sampler pressure was not significant for other aerosol setups because all sampler pressures fell within normal range.

The regression model to predict SF from RH, temperature, and sampler pressure was not significant for Rift Valley Fever Virus with the Collison or the Aeroneb. Thus, RH and temperature do not play a role in the improved aerosol performance observed with the Aeroneb for RVFV. Sampler pressure was within normal range for all RVFV aerosols, and thus did not affect aerosol performance.

In the nose-only tower, the Collison had better aerosol performance than the Aeroneb, while in the whole-body and head-only chambers, the Aeroneb had better aerosol performance. Prior studies indicate aerosol performance of LVS is improved with higher humidity, and the multiple regression analysis from this study supports this data. The Collison was able to produce higher humidity than the BLAM and the Aeroneb in the NOT, and the Aeroneb was able to achieve a humidity higher than the Collison in the RWB chamber. This could explain why the Collison has better aerosol performance than both alternatives in the NOT, but the Aeroneb has better aerosol performance than the Collison in the RWB chamber. For influenza and RVFV, the cause of improved aerosol performance seen in the whole-body and head-only chambers is harder to determine. Examined environmental parameters were not the cause of improved

aerosol performance for these viruses. This suggests it is the method used to produce aerosols, rather than the resulting RH or temperature, that improved aerosol performance for influenza and RVFV with the Aeroneb.

While the SF was lower for the Aeroneb in the NOT, if high infectious doses are not required for experiments, the Aeroneb may be a better choice for aerosol experiments since less sample is needed and the coefficient of variation was lower than the Collison potentially giving greater reproducibility between aerosols. These results support that there is currently no 'one size fits all' aerosol generator, and aerosol performance of different aerosol generators should be fully characterized for different microorganisms to allow for appropriate selection of aerosol equipment based on the goals of the experimental design. While the data presented here would not lend toward recommending the BLAM for the pathogens/chambers tested, the data would suggest that the Aeroneb is potentially a suitable alternative to the Collison.

5.1 PUBLIC HEALTH SIGNIFICANCE

5.1.1 Bioterrorism Defense

Biological weapons are defined as any infectious agent or toxin derived from a living organism and are just one of the many tools terrorists can use to harm the government or the population to further their political or social agenda [55, 56]. These attacks can be inflicted directly on populations or indirectly by attacking crops or farm animals [55, 56]. Infectious diseases have been used throughout history as weapons, but today developing these weapons is relatively cheap and very effective; there are many bacteria and viruses readily accessible in the environment that

could be used as biological weapons. Corpses infected with plague were catapulted over walls in 1346 during the siege of Kaffa; smallpox infested blankets were given to Indians loyal to the French during the French and Indian War; anthrax was mailed to governmental officials and the news media in 2001 [55, 56]. Even individuals can use infectious agents to harm others, such as the *Shigella dysenteriae* incident in Dallas where laboratory workers were invited to eat intentionally contaminated muffins and donuts in 1996 [55]. Despite the Geneva Protocol of 1925, many countries had offensive biological weapons programs until the development of the Biological Weapons Convention in 1975, which banned the production of bioweapons [55]. However, Iraq admitted to researching offensive use of biological weapons in 1991 and Russia in 1992 [55].

With the threat of biological attacks, the United States continues researching potential bioterrorism agents to help defend the nation against an attack through studying disease pathogenesis and developing vaccines and therapeutics. Potential biological weapons will cause high mortality, be very contagious and virulent at low doses, have predictable incubation times, and be difficult to identify early due to nonspecific symptoms. Studies indicate that aerosol will be the most likely method of attack since aerosol dissemination is not easily detectable, hard to defend against, and has the potential to reach millions in a short time span [55]. It is difficult to develop vaccines and therapeutics for many biological select agents and toxins due to the low incidence of disease, and since aerosolization is generally not the natural method of transmission disease pathogenesis may be different from natural routes of transmission [55]. Thus, it is vitally important for public health to perform aerosol studies in animals to study disease pathogenesis and test vaccines and therapeutics to help prevent or contain biological attacks.

5.1.2 Animal Models of Human Respiratory Infections

The use of animal models dates back to ancient Greece, where animals were used to better understand human anatomy and physiology [57]. These initial observational animal studies involved outbred animals. As the use of animal models transitioned into experimental use, the need to control genetic variability became apparent [57]. Individuals such as Clarence Little began inbreeding mice to reduce genetic variability, eventually reaching the ability to breed genetically identical mice [57]. Advances in the field of genetics, such as the Cre-Lox system, allow the manipulation of animal genomes to create specific disease models that are not naturally available [57].

The Animal Efficacy Rule allows the FDA to rely on evidence produced from animal studies rather than human clinical trials to determine the effectiveness of vaccines or therapeutics [9-11]. Data from animal studies can be used if the mechanism of toxicity is understood, if the desired effect is shown in animal models expected to correlate with the human response, if the study end point is related to the desired effect in humans, and if effective dose can be predicted from pharmacokinetic and pharmacodynamic data [9-11]. The implementation of the FDA Animal Rule increased the importance of animal models in vaccine and therapeutic development. Laboratory accidents in the early 20th century demonstrated that pathogens not normally spread through aerosols, such as alphaviruses, can cause disease through the respiratory route when aerosolized. Subsequently, these agents were evaluated as potential offensive biological weapons by the United States and former Soviet Union [6]. Therefore the disease course and virulence in humans for many of these pathogens after inhalation is not known, and thus developing animal models is vitally important for understanding disease pathogenesis and developing therapeutics and vaccines [6].

Differences in anatomy, mucocilliary clearance, or route of administration could lead to variances in disease pathology in animal models of respiratory illnesses [57, 58]. Differences inherent in the model can be controlled by careful selection of animals that exhibit characteristics needed for disease progression. Ferrets are good models for influenza because their respiratory tract is shaped similar to the human respiratory tract and is marked by a similar sialic acid receptor distribution as in humans [58]. Other confounding factors can be controlled through a thorough understanding of experimental design and the effects that different parameters can have on disease outcome. Route of administration, particle size, and dose are all factors which can affect disease progression in animal models and need to be controlled [13, 21]. Thus, aerosol research methods and equipment need to be well characterized to optimize the development of animal models for diseases spread through the respiratory route [58].

BIBLIOGRAPHY

1. Roy, C.J. and D.K. Milton, *Airborne transmission of communicable infection-the elusive pathway*. 2004, DTIC Document.
2. Tellier, R., *Review of aerosol transmission of influenza A virus.* Emerg Infect Dis, 2006. **12**(11): p. 1657-1662.
3. Fernstrom, A. and M. Goldblatt, *Aerobiology and its role in the transmission of infectious diseases.* Journal of pathogens, 2013. **2013**.
4. Wells, W.F., *Airborne Contagion and Air Hygiene. An Ecological Study of Droplet Infections.* Airborne Contagion and Air Hygiene. An Ecological Study of Droplet Infections., 1955.
5. Mizgerd, J.P., *Lung infection—a public health priority.* PLoS Med, 2006. **3**(2): p. e76.
6. Roy, C., D. Reed, and J. Hutt, *Aerobiology and inhalation exposure to biological select agents and toxins.* Veterinary Pathology Online, 2010. **47**(5): p. 779-789.
7. Bosch, A.A., et al., *Viral and bacterial interactions in the upper respiratory tract.* PLoS Pathog, 2013. **9**(1): p. e1003057.
8. Roy, C.J. and M.L. M. Pitt, *Infectious Disease Aerobiology: Aerosol Challenge Methods*, in *Biodefense Research Methodology and Animal Models*, J.R. Swearengen, Editor. 2012, CRC Press. p. 65-79.
9. Aebersold, P., *FDA experience with medical countermeasures under the animal rule.* Advances in preventive medicine, 2011. **2012**.
10. Snoy, P., *Establishing Efficacy of Human Products Using Animals The US Food and Drug Administration's "Animal Rule"*. Veterinary Pathology Online, 2010. **47**(5): p. 774-778.
11. Burns, D.L., *Licensure of vaccines using the Animal Rule.* Current opinion in virology, 2012. **2**(3): p. 353-356.
12. Food and Drug Administration, *Guidance for Industry: Product Development Under the Animal Rule*. 2014.
13. Bodewes, R., et al., *Pathogenesis of Influenza A/H5N1 virus infection in ferrets differs between intranasal and intratracheal routes of inoculation.* The American journal of pathology, 2011. **179**(1): p. 30-36.
14. Driscoll, K.E., et al., *Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations.* Toxicological Sciences, 2000. **55**(1): p. 24-35.

15. Berendt, R., *Relationship of method of administration to respiratory virulence of Klebsiella pneumoniae for mice and squirrel monkeys.* Infection and immunity, 1978. **20**(2): p. 581.
16. Lackemeyer, M.G., et al., *ABSL-4 aerobiology biosafety and technology at the NIH/NIAID integrated research facility at Fort Detrick.* Viruses, 2014. **6**(1): p. 137-150.
17. *AeroMP Management Platform General Description*. Biaera Technologies, LLC.
18. *Collison Nebulizer - Instructions*. 2002, BGI, Inc.: Waltham, MA.
19. May, K., *The Collison nebulizer: description, performance and application.* Journal of Aerosol Science, 1973. **4**(3): p. 235-243.
20. *Collison Nebulizer*. [cited 2016 February 25]; Available from: http://chtechusa.com/products_tag_lg_collison-nebulizer.php.
21. Thomas, R., et al., *Influence of particle size on the pathology and efficacy of vaccination in a murine model of inhalational anthrax.* Journal of medical microbiology, 2010. **59**(12): p. 1415-1427.
22. Zhen, H., et al., *A systematic comparison of four bioaerosol generators: Affect on culturability and cell membrane integrity when aerosolizing Escherichia coli bacteria.* Journal of Aerosol Science, 2014. **70**: p. 67-79.
23. Hess, D.R., *Nebulizers: principles and performance.* Respiratory care, 2000. **45**(6): p. 609.
24. Faith, S.A., et al., *Growth conditions and environmental factors impact aerosolization but not virulence of Francisella tularensis infection in mice.* Front Cell Infect Microbiol, 2012. **2**: p. 126.
25. Thomas, R.J., et al., *The cell membrane as a major site of damage during aerosolization of Escherichia coli.* Applied and environmental microbiology, 2011. **77**(3): p. 920-925.
26. Reed, D.S., et al., *Differences in aerosolization of Rift Valley fever virus resulting from choice of inhalation exposure chamber: implications for animal challenge studies.* Pathogens and disease, 2014. **71**(2): p. 227-233.
27. *Blaustein Atomizer (BLAM) Multi-jet Model*. CH Technologies (USA).
28. Zhang, G., A. David, and T.S. Wiedmann, *Performance of the vibrating membrane aerosol generation device: aeroneb micropump nebulizer™.* Journal of Aerosol Medicine, 2007. **20**(4): p. 408-416.
29. CH Technologies (USA). *Nose-Only Inhalation Exposure Systems*. [cited 2016 February 24]; Available from: http://chtechusa.com/products_inhalation-noseonly.php.
30. Roy, C.J., et al., *Impact of inhalation exposure modality and particle size on the respiratory deposition of ricin in BALB/c mice.* Inhalation toxicology, 2003. **15**(6): p. 619-638.
31. Gater, S.T., et al., *Host stress and immune responses during aerosol challenge of Brown Norway rats with Yersinia pestis.* Front. Cell. Inf. Microbio, 2012. **2**(147): p. 10.3389.
32. Verreault, D., S. Moineau, and C. Duchaine, *Methods for sampling of airborne viruses.* Microbiology and Molecular Biology Reviews, 2008. **72**(3): p. 413-444.
33. Saini, D., et al., *Sampling port for real-time analysis of bioaerosol in whole body exposure system for animal aerosol model development.* Journal of pharmacological and toxicological methods, 2011. **63**(2): p. 143-149.
34. Dabisch, P., et al., *Comparison of the efficiency of sampling devices for aerosolized Burkholderia pseudomallei.* Inhalation toxicology, 2012. **24**(5): p. 247-254.

35. Skinner, J.A., et al., *Immunologic Characterization of a Rhesus Macaque H1N1 Challenge Model for Candidate Influenza Vaccine Assessment.* Clinical and Vaccine Immunology, 2014: p. CVI. 00547-14.
36. Steel, J., P. Palese, and A.C. Lowen, *Transmission of a 2009 pandemic influenza virus shows a sensitivity to temperature and humidity similar to that of an H3N2 seasonal strain.* Journal of virology, 2011. **85**(3): p. 1400-1402.
37. Dennis, D.T., et al., *Tularemia as a biological weapon: medical and public health management.* Jama, 2001. **285**(21): p. 2763-2773.
38. Adamovicz, J.J. and D.M. Waag, *Tularemia*, in *Biodefense Research Methodology and Animal Models*, J.R. Swearengen, Editor. 2012, CRC Press. p. 147-177.
39. *Tularemia: Statistics*. 2015 October 26, 2015 [cited 2016 February 26]; Available from: http://www.cdc.gov/tularemia/statistics/index.html.
40. *Notes from the Field: Increase in Human Cases of Tularemia - Colorado, Nebraska, South Dakota, and Wyoming, January-September 2015*. Morbidity and Mortality Weekly Report 2013 December 4, 2015 [cited 2016 February 26]; Available from: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6447a4.htm.
41. Burke, D.S., *Immunization against tularemia: analysis of the effectiveness of live Francisella tularensis vaccine in prevention of laboratory-acquired tularemia.* Journal of Infectious Diseases, 1977. **135**(1): p. 55-60.
42. Cox, N. and K. Subbarao, *Global epidemiology of influenza: past and present.* Annual review of medicine, 2000. **51**(1): p. 407-421.
43. Clark, N.M. and J. Lynch 3rd. *Influenza: epidemiology, clinical features, therapy, and prevention*. in *Seminars in respiratory and critical care medicine*. 2011.
44. Swayne, D. and D. Suarez, *Highly pathogenic avian influenza.* Revue Scientifique et Technique-office International des Epizooties, 2000. **19**(2): p. 463-475.
45. Hurt, A., et al., *Susceptibility of highly pathogenic A (H5N1) avian influenza viruses to the neuraminidase inhibitors and adamantanes.* Antiviral research, 2007. **73**(3): p. 228-231.
46. Yang, Y., et al., *Detecting human-to-human transmission of avian influenza A (H5N1).* Emerg Infect Dis, 2007. **13**(9): p. 1348-1353.
47. Chan, P.K., *Outbreak of avian influenza A (H5N1) virus infection in Hong Kong in 1997.* Clinical Infectious Diseases, 2002. **34**(Supplement 2): p. S58-S64.
48. Shope, R., C. Peters, and F. Davies, *The spread of Rift Valley fever and approaches to its control.* Bulletin of the World Health Organization, 1982. **60**(3): p. 299.
49. Pepin, M., et al., *Rift Valley fever virus (Bunyaviridae: Phlebovirus): an update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention.* Veterinary research, 2010. **41**(6): p. 61.
50. *Select Agents and Toxins List*. [cited 2016 February 25]; Available from: http://www.selectagents.gov/SelectAgentsandToxinsList.html.
51. Martin, V., et al., *The impact of climate change on the epidemiology and control of Rift Valley fever.* Revue Scientifique et Technique, Office International des Epizooties, 2008. **27**(2): p. 413-426.
52. Reed, L.J. and H. Muench, *A simple method of estimating fifty per cent endpoints.* American journal of epidemiology, 1938. **27**(3): p. 493-497.
53. Goodlow, R.J. and F.A. Leonard, *Viability and infectivity of microorganisms in experimental airborne infection.* Bacteriological reviews, 1961. **25**(3): p. 182.

54. Mubareka, S., et al., *Transmission of influenza virus via aerosols and fomites in the guinea pig model.* Journal of Infectious Diseases, 2009. **199**(6): p. 858-865.
55. Anderson, P.D. and G. Bokor, *Bioterrorism: pathogens as weapons.* Journal of pharmacy practice, 2012. **25**(5): p. 521-529.
56. Beeching, N.J., et al., *Biological warfare and bioterrorism.* British Medical Journal, 2002. **324**(7333): p. 336.
57. Ericsson, A.C., M.J. Crim, and C.L. Franklin, *A brief history of animal modeling.* Missouri medicine, 2013. **110**(3): p. 201.
58. National Research Council (US) Committee on Animal Models for Testing Interventions Against Aerosolized Bioterrorism Agents, *Overcoming Challenges to Develop Countermeasures Against Aerosolized Bioterrorism Agents: Appropriate Use of Animal Models*. 2006: National Academies Press (US).