Inactivation of porcine reproductive and respiratory syndrome (PRRS) virus

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Inactivation of porcine reproductive and respiratory syndrome (PRRS) virus under static and dynamic conditions using ultraviolet (UV254) light

by

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Dedication

This thesis is dedicated to my family, who offered me unconditional love and support throughout the course of my studies.

To my wife, Sara Cutler: who has been a great source of motivation and has supported my desire to further my education despite the sacrifices. I love you.

To my children: Jordan Cutler and Caleb Cutler, who are the reason to try and make our lives better. I thank you for sacrificing to allow me complete this work.

To my parents: Dean and Jerry Cutler, who taught me the value of education and who made sacrifices for us, their children, so that we could have the opportunities they did not have.

To Drs. Thacker, Hoff, Blitvich, Hurd, and Zimmerman: Thank You.
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The question addressed in this dissertation is whether ultraviolet could be used to inactivate airborne PRRS virus. Specifically, the aim of this research was to determine if ultraviolet could be used to prevent the airborne transmission of PRRS virus. This problem was addressed in the logical series of experiments described below.

Chapter 2 addressed the problem of quantifying the concentration of airborne infectious PRRS virus at levels sufficient to infect pigs, but below the threshold of microinfectivity assays. This study explored the application of the “continuous-stirred tank reactor (CSTR) model” to this problem and validated the CSTR approach using rhodamine B dye as a surrogate for aerosolized microbial pathogens in a dynamic aerosol toroid (DAT).

In Chapter 3, the median infectious dose (ID$_{50}$) of PRRS virus isolate MN-184 via aerosol exposure was estimated. This information was needed to determine the level of ultraviolet inactivation necessary to stop transmission of PRRS virus. Infection occurred at virus concentrations too low to quantify by microinfectivity assays. Therefore, exposure dose was determined using two indirect methods ("calculated" and "theoretical"). "Calculated" virus dose was derived from the concentration of rhodamine B monitored over the exposure sequence. "Theoretical" virus dose was based on the continuous stirred-tank reactor model. The estimation of ID$_{50}$ was modeled on the proportion of pigs that became infected using the probit and logit link functions for both "calculated" and "theoretical" exposure doses.

Chapter 4 established UV$_{254}$ inactivation constants for: influenza virus Type A, porcine respiratory and reproductive syndrome virus (PRRS virus), bovine virus diarrhea virus (BVDV), and reovirus. Viruses were exposed to various UV$_{254}$ doses then titrated for infectious virus. Analysis showed that virus inactivation by UV$_{254}$ was more accurately described by a two-stage inactivation model, rather than the traditional one-stage inactivation model. These results provided insight into the dose of ultraviolet required to inactivate airborne pathogens (Chapter 5).
Chapter 5 described the effects of temperature and relative humidity on the inactivation of PRRS virus by ultraviolet. Viral aerosols were exposed to four doses of UV$_{254}$ under three temperature ranges and three relative humidity ranges. This study allowed for calculating the dose of UV$_{254}$ required to inactivate airborne PRRS virus under various conditions.
CHAPTER 1. REVIEW OF ULTRAVIOLET AND THE MECHANISMS UNDERLYING THE INACTIVATION OF INFECTIOUS AGENTS BY ULTRAVIOLET IRRADIATION

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Timothy D. Cutler, Jeffrey J. Zimmerman

ABSTRACT

We review the principles of ultraviolet (UV) irradiation, the inactivation of infectious agents by UV, and current applications for the control of microorganisms. In particular, wavelengths between 200nm and 280nm (germicidal UV) affect the double-bond stability of adjacent carbon atoms, including pyrimidines, purines, and flavin. Thus, the UV inactivation of microorganisms results from the formation of dimers in RNA (uracil and cytosine) and DNA (thymine and cytosine). The classic application of UV is the inactivation of microorganisms in biological safety cabinets. In the food-processing industry, germicidal UV has shown potential for the surface disinfection of fresh-cut fruit and vegetables. UV treatment of water (potable and wastewater) is increasingly common because the process is effective against a wide range of microorganisms, overdose is not possible, chemical residues or by-products are avoided, and water quality is unaffected. UV has been used to reduce the concentration of airborne microorganisms in limited studies, but the technology will require further development if it is to gain wider application. For bioaerosols, the primary technical challenge is delivery of sufficient UV to large volumes of air, but the absence of UV inactivation constants for airborne pathogens under a range of environmental conditions (temperature, relative humidity) further compound the problem.
INTRODUCTION
The purpose of this review is to provide an overview of ultraviolet and its application for the control of microorganisms. The first work on the inactivation of microorganisms by ultraviolet light was published in 1892 (Ward, 1892), with the "germicidal" effects of ultraviolet wavelengths reported in 1903 (Barnard and Morgan, 1903). In 1927, Rivers and Gates used ultraviolet to inactivate virus in solution and proved the efficacy of the method through subcutaneous inoculation of rabbits. The use of ultraviolet to inactivate microorganisms in the environment began when Wells and Wells (1938) described the use of ultraviolet to inactivate microorganisms in hospital operating rooms. Wheeler et al. (1945) used ultraviolet to "disinfect" Army and Navy barracks for the control of airborne rubella virus and Streptococcus pyogenes and in 1947, the use of ultraviolet irradiation reportedly reduced the spread of airborne "measles" in classrooms (Perkins et al., 1947). In 1961, Riley demonstrated the efficacy of ultraviolet for the control of airborne tuberculosis by showing that untreated ventilated air from wards housing infectious TB patients produced infection in guinea pigs whereas air irradiated with ultraviolet light did not. Thereafter, the research moved from qualitative to quantitative measures of the effect of ultraviolet on microorganisms. In one of the first papers to quantify the genetic damage induced by ultraviolet, Miller and Plagemann (1974) calculated that a dose of 7mJ per cm² induced the formation of 1.7 uracil dimers per mengovirus plaque-forming unit and that dimer formation increased the production of empty viral capsids, altered protein structures, and increased RNase activity. Four years later (1978), Sarasin and Hanawalt reported that a 10mJ per cm² dose resulted in 7 pyrimidine dimers per simian virus 40 (SV40) genome (Kowalski, 2009). With the exception of water treatment applications, relatively little research on the effect of ultraviolet on microorganisms was published from the mid-1970s to 2000. However, concerns for bioterrorism, antibiotic-resistant bacteria, and airborne spread of emerging and re-emerging pathogens, e.g., pandemic influenza virus and severe acute respiratory syndrome (SARS) coronavirus, have stimulated renewed interest in the use of ultraviolet as a microbial inactivant (Walker and Ko, 2007).
THE ULTRAVIOLET SPECTRUM

The electromagnetic spectrum includes energies possessing both electrical and magnetic properties. Classified by wavelength and photonic interaction with matter (ionizing or non-ionizing), these wavelengths vary from very high vibrational energy ($1 \times 10^{-7}$ nm) to infinitely long wavelengths and encompass all known forms of energy, from high energy gamma rays ($3 \times 10^{-3}$ nm), microwaves to lower energy radio waves ($3 \times 10^{13}$ nm) (Van Heuvelen, 1982). Ultraviolet wavelengths lie between the high energy X-rays ($\leq 100$ nm) and the lower energy visual spectrum ($> 400$ nm). Wavelengths longer than 300nm begin the visible spectrum of the electromagnetic spectrum, followed by the infrared spectrum beginning at 700nm, microwave frequencies (starting at $3 \times 10^{6}$ nm), and the radar/radio frequencies ($3 \times 10^{8}$ nm and beyond). There is no clear delineation between x-rays and the ultraviolet spectrum; rather it is the nature of their interaction with matter that best defines the end of x-rays and the beginning of the ultraviolet spectrum. The energy-matter interactions of wavelengths less than 100nm result in ionization (a change in atomic charge) of the exposed matter. As the wavelengths increase, the energy-matter interaction results in less ionization and more electron excitation (electrons jumping to higher energy levels) as the energy is absorbed by molecules.

Although there are several classification schemes (Jagger, 1967), the ultraviolet spectrum may be most simply divided into four general classifications based on the wavelength's interaction with molecules: (1) "vacuum ultraviolet" (VUV), (2), ultraviolet “C” (UVC), (3) ultraviolet “B” (UVB), and (4) ultraviolet “A” (UVA). The VUV spectrum includes wavelengths $<200$ nm. The most energetic wavelengths within the ultraviolet spectrum, VUV readily interacts with oxygen atoms and their interaction with organic molecules is detrimental even at low doses, however these wavelengths exist only in a vacuum due to the high energy. UVC encompasses wavelengths between 200nm and 280nm. This spectrum is also called the “germicidal” spectrum because of its biocidal effects on bacteria (Jagger, 1967). UVB ranges from 280nm to 315nm and is the wavelength responsible for "sun burning" the skin and the synthesis of Vitamin D (Goodsell, 2001). UVA ranges from 315nm to 400nm and is the primary light produced by black light fixtures (Stowe, 2005).
Both UVA and UVB are used in industry, e.g., to activate organic polymers used in laminates, production of medical devices (Stowe, 2005). UVA and UVB, but not UVC, wavelengths, are long enough to pass through the earth’s atmosphere to the earth’s crust and can penetrate a short distance into the world’s oceans (Jagger, 1967).

The focus of this review is UVC and its effect on microorganisms. UVC is sometimes termed "ultraviolet germicidal irradiation" (UVGI) to distinguish it from the non-germicidal wavelengths, UVA and UVB (Kowalski, 2009). UVC is biologically important because unsaturated organic compounds, i.e., compounds that are not fully saturated with hydrogen atoms or are composed of conjugated bonds, efficiently absorb wavelengths between 200nm and 280 nm (Jagger, 1967). Conjugated bonds hold two electron pairs, each electron in the pair possesses an independent and opposite spin of equal energy. When a photon of ultraviolet radiation energy strikes an electron, it is induced to rise to an excited (higher energy) level. This disruption of stable electrons can travel the entire organic structure, raising a bonded electron out of a bonding pair, and result in an unstable conformation. In conjugated bond-containing structures, the entire structure acts as a chromophore, i.e., the \( \pi \) orbitals are shared throughout the ringed structure (Jagger, 1967). Because of this, the entire structure absorbs the ultraviolet energy (photon) and this extra energy is drained off into the weakest bond, thereby causing conformational changes to occur (Jagger, 1967).

Conjugated organic structures include nitrogen-containing ring structures such as pyridines, pyrimidines, flavins, and the aromatic amino acids (Jagger, 1967). Because these structures act as chromophors, these wavelengths can also be used for nucleic acid analysis. This process is based on the difference in absorption between nucleic acids and proteins. Essentially, this methodology relies on that fact that peptide bond exhibits double-bond characteristics, whereas aromatic amino acids absorb ultraviolet (Jagger, 1967). Figure 1 shows the structure of conjugated bases within genetic material. Figure 2 shows the structure of conjugated bases within the aromatic amino acids.
Figure 1. UV\textsubscript{254}-labile organic structures in nucleic acids. (Cacycle, 2007. Property in public domain downloaded June 2010.)

Figure 2. UV\textsubscript{254}-labile organic amino acids.

**MEASURING ULTRAVIOLET: RADIOMETRY AND ACTINOMETRY**

Measurement is the heart of science and the basis upon which effects are evaluated. There are two basic ways to measure the intensity and duration of ultraviolet radiation: radiometry and actinometry.

Radiometry measures irradiance, the ultraviolet energy striking a surface from all forward angles at a point in time expressed as energy (watts) per unit area (Bolton and Linden, 2003). A radiometer is a sensor with an electronic readout device that displays the sensor readings. Sensors are wavelength-specific and have a cosine response that accounts for the incident
angle of the light source as it strikes the sensor’s surface. (Note: to ensure accurate ultraviolet measurements, radiometers need to be calibrated annually.) Since ultraviolet sensors typically have a measurement area of one square centimeter, the amount of ultraviolet energy arriving at the surface is commonly measured in watts per m² or mW per cm². In biological experiments it is necessary to account for both the intensity of the ultraviolet light energy (irradiance) and the length of exposure (time). Therefore, radiometers are often equipped to measure cumulative exposure over time (mWs per cm²).

Actinometry is a second method of measuring ultraviolet light energy. Actinometry is based on chemical systems that undergo light-induced reactions at specific wavelengths for which the quantum yield is accurately known (Kuhn et al., 2004). The quantum yield is a measure of molecular UV-absorption efficiency of a chemical and is described as the ratio of the number of chemical changes per unit time to the number of photons absorbed per unit time (Kuhn et al., 2004):

\[ \Phi = \frac{N_c}{N_p} \]  

(1)

Where:
- \( \Phi \) = Quantum yield
- \( N_c \) = Number of molecules chemically reacting
- \( N_p \) = Number of photons absorbed

For example, iodide/iodate solution is an actinometer commonly used in the UVC spectrum because it absorbs energy between 200nm and 300nm. The concentration of the product (triiodide) is directly proportional to the intensity, i.e., the number of photons absorbed, as measured on a spectrophotometer at 352nm (Rahn et al., 2005). This is not to be confused with the radiometric ultraviolet dose which is independent of photon absorbance.

There are important differences between the two methods and it should be recognized that each provides results based upon different parameters. Actinometry is the preferred method
of measuring ultraviolet exposure in the field of photochemistry and photochemists express ultraviolet exposure in units of quantum yield. Actinometry is neither convenient nor practical in photobiology and photobiologists measure ultraviolet using radiometry and express exposure in joules per cm$^2$. This difference in the expression of ultraviolet exposure units presents a fundamental problem in communicating results across disciplines.

Ultraviolet is also sometimes described in terms of fluence rate, the energy (mW) passing through a cross-sectional area (cm$^2$), with ultraviolet dose defined as the fluence rate per unit time in seconds (s), i.e., mWs per cm$^2$ (Bolton, 2000). A joule is expressed as energy × time, therefore ultraviolet dose is expressed as joules per cm$^2$. Because radiometers are in common use and more readily implemented than chemical actinometry, the term "ultraviolet dose" is sometimes used interchangeably with "fluence rate", regardless of the object's ability to absorb ultraviolet. Therefore, depending upon the field or discipline, the terminology used may not strictly conform to the definitions of the International Union of Pure and Applied Chemistry Working Party on Ultraviolet Disinfection (Bolton and Linden, 2003).

CONSIDERATIONS IN EXPERIMENTAL UV254 PHOTOBIOLOGY

Photobiology describes the interaction between light and living matter. Bolton and Linden (2003) outlined the basic requirements in the design of bench scale ultraviolet inactivation apparatus for wastewater experimentation, but these standards also apply to other types of ultraviolet experimentation involving inactivation of microorganisms. Specifically, bench-scale ultraviolet inactivation experiments should be reproducible across laboratories and contain the basic elements of good experimental design. Results that provide a foundation for forward progress will also use accepted terminology to describe the experimental design and results. Thus, the terminology presented in this review is based on the recommendations of the International Union of Pure and Applied Chemistry Working Party on Ultraviolet Disinfection (Bolton and Linden, 2003).
The single most significant physical factor in UV$_{254}$ inactivation is lamp design and performance (VanOsdell and Foarde, 2002). In microbiology, the majority of work involving photobiology involves "germicidal" UVC, with UV$_{254}$ being the wavelength considered to possess the strongest inactivating effect. Importantly, VanOsdell and Foarde (2002) noted that for a UV$_{254}$ system to efficiently inactivate pathogens, the bulb design (type of quartz, internal gas composition, operating temperature, ballast type) must be matched with the environmental conditions (temperature and relative humidity).

The first mercury vapor arc lamp was developed by Wheatstone in 1835 (Kowalski, 2009). Other metallic gases (zinc, iron, or xenon) can be energized into ultraviolet emission, but excited mercury gas is the most efficient ultraviolet emitter and is used extensively in UV$_{254}$ bulbs. Mercury gas ultraviolet emitters are produced as low-, medium-, and high-pressure bulbs. Low- and medium-pressure mercury lamps consist of electrodes that produce electrons that collide with mercury atoms causing them to emit photons, predominately at 253.7 nm (Jagger, 1967). High-pressure mercury lamps, while similar in design, use electrodes capable of high voltage, thus allowing for an increase in the efficiency of emitted photons (Jagger, 1967).

Low-pressure bulbs (internal pressure of less than 1 bar) operate at a low surface temperature and emit monochromatic (UV$_{254}$) wavelengths (Figure 3). Medium-pressure bulbs (internal pressure slightly higher than 1 bar) operate at higher surface temperature and emit polychromatic light (Figure 3). To eliminate undesired ultraviolet wavelengths, the synthetic quartz containing the vaporized mercury atoms can be treated with wavelength-dependant ultraviolet-absorbing components that block specific wavelengths from exiting the bulb. Proper temperature control must be maintained when using medium-pressure vapor lamps because the lamps produce heat and the output (irradiance) of these lamps is temperature-sensitive. As a result, these lamps require additional power to increase the internal temperature and pressure of the lamp. This increased temperature allows for an increased ultraviolet light spectrum, but until the bulbs reach operating temperature, their output fluctuates. For that reason, under experimental conditions, a shutter system is needed so that
light-emitting bulbs reach peak performance prior to target exposure (Bolton and Linden, 2003).

Under experimental conditions, the structure of the ultraviolet-emitting apparatus must be designed to provide a spatially homogeneous field of irradiation. This can be accomplished through the use of a collimating tube (Bolton and Linden, 2003; Shen et al., 2005; Thurston-Enriquez et al., 2003). However, depending on the experiment, a collimating tube may not be required. That is, in the absence of reflected ultraviolet energies and with irradiance from one plane only, the cosine sensors will account for any incident irradiation. Sensors for measuring ultraviolet and holding treatment samples should be secured to a thermally and physically stable exposure stage. For accurate measurements of ultraviolet exposure, sensors must be placed at the same distance from the energy source as the irradiated sample and absorbance of ultraviolet by the sample matrix must be taken into account. Bolton and Linden (2003) consider it necessary to stir the solution to ensure uniform ultraviolet dose, but small volumes or a matrix that does not readily absorb ultraviolet need not be stirred.
**Lambert-Beer law**  
In brief, this law states that some of the UV254 energy to which the target is exposed will be absorbed by the surrounding environment and this absorption must be taken into account. For microorganisms in suspension (liquid or aerosol), the average UV254 intensity may be calculated as follows (Thurston-Enriquez et al., 2003):

\[ I_{\text{average}} = \frac{I_0(1 - e^{-a_\text{e}L})}{a_\text{e}L} \]

(2)

Where:

- \( I_{\text{average}} \) = average UV254 intensity (milliwatts per square centimeter)
- \( a_\text{e} \) = absorbance of the virus suspension to the base e
- \( I_0 \) = UV254 intensity after passing through solution
- \( L \) = depth (centimeters) of the solution irradiated by the UV254 energy.

For liquid media, the amount of ultraviolet energy absorbed by the solution can be determined by measuring the amount of ultraviolet that passes through the matrix using a quartz cuvette equal to the depth of the sample (Lambert-Beer’s Law). Jagger (1967) provides information on the absorption coefficients for various solutions, but only empirical data will provide the researcher the information to necessary to determine if stirring is necessary.

**MECHANISM OF ULTRAVIOLET INACTIVATION**

Inactivation of microorganisms by ultraviolet is initiated at the quantum level. The quantum yield is the number of photons, or the photon density, impacting a surface area. Each photon carries an amount of energy called a quantum (\( \varepsilon \)) determined from quantum mechanics (Jagger, 1967, and Kowalski, 2009).
ε = (h)(ν) \hspace{1cm} (3)

Where:
- \( ε \) = Energy in one photon
- \( h \) = Planck’s constant, \( 6.626 \times 10^{-34} \) joules (J)
- \( ν \) = Frequency in hertz (Hz)

Much of the quantal information was determined in the 1960s, when ultraviolet researchers focused on the mechanism of inactivation. During this time, researchers estimated the quantum yields required for dimerization of nucleic acids (Kleczkowski, 1963; Shore, 1956). Kowalski (2009) calculated that ultraviolet at a wavelength of 253.7 nm has a frequency of \( 1.18 \times 10^{15} \) Hz and \( 7.819 \times 10^{19} \) J of energy per photon. It follows that each joule contains \( 1.279 \times 10^{18} \) photons and an ultraviolet dose of one millijoule per cm\(^2\) will produce \( 1.279 \times 10^{15} \) photons per cm\(^2\). Thus, a microorganism with a diameter of 0.1 micron, i.e., a cross-sectional area of \( 3.14 \times 10^{-14} \) m \( (3.14 \times 10^{-12} \) cm), will be subjected to the passage of approximately 401,000 photons per second (Kowalski, 2009).

Ultraviolet energy at 254nm readily affects the double-bond stability between adjacent carbons. There are two types of molecular bonds occurring in conjugated organic structures, the sigma (\( σ \)) orbitals and the Pi (\( π \)) orbitals. The higher energy sigma orbitals (shorter wave function) are located closer to the nucleus of the two bonded atoms. The Pi orbitals are of lower energy and are nonlocalized about the bonded pair. The lower energy Pi orbitals are more stable and, therefore, have longer wave function (Kowalski, 2009). Conjugated ring structures, like pyrimidines, purines, and aromatic amino acids have large, non-localized Pi orbitals (Smith and Hanawalt, 1969). When an incoming UV\(_{254}\) photon strikes a Pi orbital, the photon’s energy is converted to vibrational energy (Kowalski, 2009). If this vibrational energy is sufficient, the Pi orbital is pushed into a transient unstable state that exists for a femtosecond \( (10^{-15}) \). This unstable state must return to the ground state either by dissipation of the energy or through modification of the bond by rotation (Kowalski, 2009).
Unsaturated organic compounds are essential to cell reproduction and cell metabolism. Unsaturated organic compounds vulnerable to UV254 inactivation include pyrimidines, purines, and flavin. Pyrimidines provide the basic structure for nucleobases uracil (a component of tRNA), thymine (a component of DNA and tRNA), and cytosine (a component of DNA and RNA). Purines provide the basic structure for nucleobases adenine and guanine (in DNA and RNA) and the aromatic amino acids, phenylalanine and tyrosine. Flavin is an unsaturated organic compound found in the aromatic amino acid tryptophan and is the basic structure of flavin adenine dinucleotide (FAD), a molecule necessary for metabolic redox reactions.

Nucleic acids are composed of bases, sugars and phosphates. Photons affect DNA and RNA by inducing molecular transformation, i.e., photoproducts, of the genetic material. The sugars and phosphate groups do not absorb wavelengths above 210nm, but conjugated bases have peak absorption of ultraviolet light energy at 260nm, with pyrimidines being 10 times more sensitive to UV254 than purines (Jagger, 1967). It follows that uracil and cytosine in RNA and thymine and cytosine in DNA are the targets of UV254 inactivation. Whether the target is RNA or DNA, the mechanism of UV254 inactivation is hydration of the base or base dimerization (Jagger, 1967). There are 6 possible photoproducts induced by ultraviolet: (1) thymine-thymine dimer, (2) cytosine-cytosine dimer, (3) cytosine-thymine dimer, (4) uracil-uracil dimer, (5) uracil-thymine dimer, and (6) uracil-cytosine dimer (Kowalski, 2009). The photoproducts requiring the least energy are the thymine-complex dimer and the uracil-complex dimer (Jagger, 1967; Kowalski, 2009). Cytosine hydrate, another UV254-induced photoproduct, occurs in RNA and single-stranded DNA (Smith and Hanawalt, 1969). This structure requires more energy, but is formed when UV254 irradiation of cytosine yields 6-hydroxy-5,6-dihydrocytine (O'Donnell et al., 1994). In DNA, the thymine dimer is the photoproduct with the highest quantum yield (Kowalski, 2009). These dimers occur when the hydrogen bonds linking the thymine bases are lost and the respective 5 and 6 carbon atoms are cross-linked. A representation of a generic dimer is shown in Figure 4.
The biological effects of UV$_{254}$ exposure are reversible. In bacterial cells, dimer formation is reversible via absorption of wavelengths between 300nm and 500nm (photo reactivation) or by photolyase enzymes that split the dimers. DNA viruses utilize host cellular polymerase enzymes to excise dimers and replace the damaged DNA (Kowalski, 2009). Generally, viruses do not produce their own photolyases with the exception of fowlpox virus, the only virus known to code for its own photolysase enzyme production (Srinivasan et al., 2001).

Figure 4. **Dimer formation between adjacent pyrimidines or purines nucleobases.** Illustration by D. Herring (http://earthobservatory.nasa.gov/Library/UVB).

The ultraviolet inactivation of microorganisms can be achieved with either monochromatic or polychromatic emitters. Monochromatic lamps producing primarily UV$_{254}$ are routinely used to inactivate microorganisms. Compared to monochromatic lamps, polychromatic lamps may possess greater efficiency (Linden et al., 2007). For example, Eischeid et al. (2009) reported low-pressure monochromatic ultraviolet lamp doses of 30mJ per cm$^2$, 50 mJ per cm$^2$, and 80mJ per cm$^2$ resulted in 2 log, 3 log, and 4 log reduction of adenovirus type 2 virus, respectively. In contrast, doses of 10 mJ per cm$^2$ and 25 mJ per cm$^2$ from medium-pressure polychromatic ultraviolet lamps resulted in 2.5 log and 4.5 log adenovirus reductions, respectively. Presumably these differences in inactivation reflected the fact that monochromatic ultraviolet wavelengths only caused genetic damage, whereas polychromatic wavelengths also affected aromatic proteins. That is, the structure and function of microbial
proteins depend on their primary, secondary, and tertiary structures, which reflect their constituent amino acids. It is estimated that one in 10 amino acids are susceptible to photochemical processes and, hypothetically, the photochemical alternation of any of these amino acids could affect protein structure and function (Jagger, 1967). Therefore, the efficiency of ultraviolet inactivation could be increased though the use of medium- or high-pressure polychromatic bulbs, but this gain is generally offset by the additional expense of operating this equipment.

**PRINCIPLES OF ULTRAVIOLET INACTIVATION**

The inactivation kinetics of ultraviolet can be described as a first order chemical reaction. That is, the amount of reagent (ultraviolet irradiance) will equal the amount of product (modified conjugated bonds) in a given time period. The Stark-Einstein Law states that if a photon is absorbed, then only one photon should be required for the formation of one photoproduct. This law is the foundation of the “one-hit” (first order) kinetics that have historically been used to describe UV₂₅₄ inactivation (Hiatt, 1964; Kowalski, 2009; Qualls and Johnson, 1983; Thurston-Enriquez et al., 2003).

**Grotthus-Draper Law** The Grotthus-Draper Law states that photons must be absorbed for a photochemical reaction to occur (Kowalski, 2009). Following this line of thought, Bolton and Linden (2003) suggest that the term “ultraviolet dose” should be used to describe the total energy absorbed by the target. Problematically, energy striking an object is not necessarily absorbed by the object (Jagger, 1967) and absorbed photons may not produce a photochemical reaction (Kowalski, 2009). Currently the only method to measure the absorptive efficiency of a conjugated organic molecule is actinometry.
Bunsen-Roscoe Reciprocity Law  The Bunsen-Roscoe Reciprocity Law states that microbial inactivation is dependent upon dose and dose is the product of ultraviolet intensity expressed in mW per cm² and exposure time expressed in seconds (Riley and Kaufman, 1972).

\[ D = I \times T \]  

(4)

Where:

- \( D \) = Ultraviolet dose
- \( I \) = Irradiance (intensity) in mW per cm²
- \( T \) = Exposure time in seconds

The Bunsen-Roscoe law is important and relevant to microbial inactivation because it shows that, although the ultraviolet irradiance drops as the target moves further away from the source (except in a vacuum), the desired ultraviolet dose can be achieved by increasing the exposure time. This law is fundamental because it allows for the comparison of results from experiments using different ultraviolet equipment types, wattages, and conditions, when the exposure time is known.

Chick's Law  Chick's Law states that as disinfectant contact time (t) increases, the ratio of viable microorganisms \( (N_t) \) to the total \( (N_0) \) microbes at time zero decreases. Chick's law was originally used to describe the relationship between chlorine and the inactivation of microorganisms in wastewater (Rubin and Elmaraghy, 1977). Figure 5 illustrates first order inactivation kinetics where absorbance of one photon results in inactivation.
Since the 1950s, inactivation constants (k) derived using Chick's Law have been used to measure the sensitivity of microorganisms to ultraviolet inactivation, with larger inactivation constant values (larger slope) indicative of greater susceptibility to ultraviolet inactivation.

The following equation is a modification of the formula used by Tseng and Li (2005) to solve for the inactivation constant (k).

\[ k = \frac{\log_{10} \left( \frac{N_t}{N_0} \right)}{\text{Dose}} \]  

(5)

Where:

- \( k \) = the inactivation constant
- \( N_0 \) = quantity of microbes at time zero
- \( N_t \) = quantity of microbes at UV254 exposure time "t"
- \( \text{Dose} \) = ultraviolet light dose

ONE-STAGE VS TWO-STAGE INACTIVATION

Hiatt (1964) published the first work detailing the kinetics of microbial activation and noted that the “first order” inactivation model, i.e., the single-hit hypothesis proposed by the Stark-Einstein Law and described by a single inactivation constant (k), was accurate only if the exposed viral population was homogenous and inactivation did not require cumulative
damage. Hiatt (1964) also described the classical inactivation curve dynamics of "shouldering" and "tailing". "Shouldering" refers to an increase of UV$_{254}$ dose with no corresponding increase in microbial inactivation, whereas "tailing" is a decrease in UV$_{254}$ dose with no corresponding decrease in microbial inactivation (Hiatt, 1964). In contrast to the one-stage inactivation model, the two-stage model proposes that exposure of microbial populations to an inactivant may reveal two subpopulations: one subpopulation ($f$) more susceptible to inactivation and a second subpopulation ($1-f$) more resistant (Cox, 1976; Kowalski et al., 2002; Hiatt, 1964).

\[
\log_{10} N_t = \log_{10} N_0 + \log_{10}[(1-f) \cdot 10^{-K_1 \cdot \text{Dose}_t} + f \cdot 10^{-K_2 \cdot \text{Dose}_t}]
\]  

(6)

where:

- $N_t$ = quantity of virus in the test sample after treatment with Dose$_t$
- $N_0$ = quantity of virus in the unexposed control sample,
- $f$ = the resistant fraction of the total initial virus population with inactivation rate $K_2$
- $(1-f)$ = the susceptible virus population fraction with inactivation rate $K_1$
- $k_1$ = the inactivation rate of the inactivation curve for the "fast decay population"
- $k_2$ = the inactivation rate of the inactivation curve for the "resistant population"
- Dose$_t$ = UV$_{254}$ intensity $\times$ time

In practice, the two-stage inactivation analysis should be utilized when the statistical analysis indicates that the data are better described by two-stage vs. one-stage inactivation kinetics.

**APPLICATIONS OF ULTRAVIOLET TO THE INACTIVATION OF MICROORGANISMS**

**In the laboratory** The classic application of ultraviolet is the inactivation of microorganisms in biological safety cabinets. In many laboratories, turning on the ultraviolet lamp after using the cabinet is standard operating procedure. The current version of the National Science Foundation (NSF) International Standard 49 (Section 5.25.2) does not mandate the use of ultraviolet in biosafety cabinets (Meechan and Wilson, 2006). Further,
the Centers for Disease Control and Prevention states that the use of ultraviolet in biosafety cabinets is neither recommended nor necessary (CDC, 2009). Ultraviolet inactivation of microorganisms on surfaces is ancillary to standard chemical disinfection and should not be relied upon as the sole method of disinfection.

**Food processing** In the food-processing industry, germicidal ultraviolet has shown potential for the surface disinfection of fresh-cut fruit and vegetables. In studies on carrots (Mercier et al., 1993), grapes (Nigro et al., 1998), sweet potatoes (Stevens et al., 1999), and spinach leaves (Artés-Hernéndez et al., 2009), UVC treatment was shown to reduce product deterioration and prolong storage life. Alone or in combination with ozone, ultraviolet reduced the number of viable microorganisms in water used to wash fresh-cut onion, escarole, carrot, and spinach (Selma et al., 2008). Ultraviolet may provide a viable alternative to chemical sanitizers, e.g., titanium dioxide (TiO₂) or chlorine. Currently, there is interest in developing non-thermal methods for the sterilization of juices; an objective in which UVC may play a role (Guerrero-Beltran and Barbosa-Cánovas, 2005).

**Water treatment** The application of ultraviolet to food preservation is a relatively recent development, but the use of ultraviolet for the treatment of water has an extensive history: the first system for ultraviolet treatment of potable water went into operation in Marseilles, France in 1910 (Kowalski, 2009). Ultraviolet treatment of water was not widely implemented at the beginning of the 20th century for a variety of reasons, including high operating costs, issues with equipment reliability and maintenance, and the availability of cost-effective, chemical water treatment systems (Wolfe, 1990). Ultraviolet is regarded as broadly effective against all human pathogens (bacterial, viral, and protozoal) transmitted in water (Hijnen et al., 2006) and guidelines for the treatment of waste water and potable water with ultraviolet have been established (Environmental Protection Agency, 2003). UV treatment of water (potable and wastewater) is increasingly common because the technology is readily available, the process is effective against a wide range of microorganisms, overdose is not possible, chemical residues or by-products are avoided, and water quality is unaffected (Hijnen et al., 2006; Wolfe, 1990).
Bioaerosols  A wide variety of fungal, bacterial, and viral pathogens may be transmitted by airborne droplets or droplet nuclei (Blachere et al., 2007; Tang et al., 2006). Airborne pathogens of humans include major emergent and re-emergent agents, e.g., *Mycobacterium tuberculosis*, influenza viruses, severe acute respiratory syndrome coronavirus, *Aspergillus* spp., *Legionella* spp. (Douwes et al., 2003; Escombe et al., 2007; Wong and Yuen, 2006). Likewise, some of the most economically significant pathogens of animals are transmitted via aerosols, e.g., foot-and-mouth disease virus and porcine reproductive and respiratory syndrome virus (Alexanderson et al. 2002; Hermann et al., 2009). Regardless of the actual level of risk bioaerosols present to the public, events of recent history have raised society's awareness and concern. In the market place, ultraviolet emitters designed for installation in residential air handling system are commercially available, despite the fact that data on the efficacy of these systems is sparse (Environmental Protection Agency 2006a, 2006b, 2006c, 2006d).

Ultraviolet has successfully reduced the concentration of airborne microorganisms in targeted applications. Berg et al. (1991) found that ultraviolet irradiation of air in operating rooms during surgery significantly reduced the number of viable airborne bacteria collected at the edge of the surgical site. Likewise, installation of ultraviolet in air-handling units and ventilation systems reduced the concentration of airborne bacteria and fungi in indoor air (Levetin et al., 2001; Menzies et al., 1999; Menetrez et al., 2010).

Moving beyond narrowly focused applications, the use of ultraviolet for the routine inactivation of airborne microorganisms faces severe technical challenges. In the first place, the inactivation kinetics of most airborne pathogens is not known for the range of environmental conditions (temperature and relative humidity) under which such a system would need to function. This is a significant deficit because environmental conditions are known to affect ultraviolet, e.g., as relative humidity increases, ultraviolet becomes less efficient (Ko et al., 2000; Lai et al., 2004; Peccia et al., 2001; Tseng and Li, 2005; VanOsdell and Forde, 2000; Walker and Ko, 2007). Thus, acquiring baseline exposure doses in order to target ultraviolet exposure levels is the first priority. Beyond this, delivery of the inactivating
dose uniformly and consistently to large volumes of air is a significant challenge given the current state of the technology. At present, ultraviolet inactivation of bioaerosols can only be considered one part of an overall biocontainment plan, rather than a stand-alone solution (Memarzadeh et al., 2010).

GENERAL CONCLUSIONS

The use of ultraviolet for the inactivation of microorganisms is appealing because it is a familiar, commercially-available technology that does not involve the use of chemicals. Some applications are well developed, e.g., water treatment. Some show future promise, such as applications in food processing. One highly desirable application, the routine use of ultraviolet for the inactivation of microorganisms in aerosols, will require extensive development and ultimately may only function effectively in tandem with other technologies, e.g., photocatalysis or filtration systems.

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CHAPTER 2. VALIDATION OF THE "CONTINUOUS STIRRED-TANK REACTOR" MODEL FOR ESTIMATING THE CONCENTRATION OF AIRBORNE TARGETS IN A DYNAMIC AEROSOL TOROID

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ABSTRACT

In aerobiology, dose-response studies are used to estimate the risk of infection to a susceptible host presented by exposure to a specific dose of an airborne pathogen. In the research setting, host- and pathogen-specific factors that affect the dose-response continuum can be accounted for by experimental design, but the requirement to precisely determine the dose of infectious pathogen to which the host was exposed may be challenging. By definition, quantification of viable airborne pathogens is based on the culture of microorganisms, but some airborne pathogens are transmissible at concentrations below the threshold of quantification by culture. In this paper we present an approach to the calculation of exposure dose at microbiologically unquantifiable levels using an application of the “continuous-stirred tank reactor (CSTR) model” and the validation of this approach using rhodamine B dye as a surrogate for aerosolized microbial pathogens in a dynamic aerosol toroid (DAT).

1.0 INTRODUCTION

Airborne transmission poses a major challenge to the control of human and animal pathogens. For humans, airborne transport has been linked to the transmission of Coccidioides immitis (CDC 2009), Mycobacterium tuberculosis (de la Rua-Domenech 2006), Legionella spp. (Diedern 2007), smallpox virus (Feigel et al. 2006), and a variety of other pathogenic fungi, bacteria, and viruses (Douwes et al. 2007; Fransworth et al. 2006; Nicas et al. 2005; Stärk 1999). For animals, some of the most economically significant pathogens are
transmitted in bioaerols, e.g., foot-and-mouth disease virus (Alexandersen et al. 2002), classical swine fever virus (Weesendorp et al. 2008), and porcine respiratory and reproductive syndrome virus Dee et al. 2006). Of importance to both human and animal health are major zoonotic pathogens transmitted via aerosols, including influenza virus (Loosli et al. 1943; Wong and Yuen 2006), severe acute respiratory syndrome (SARS) coronavirus (Booth et al. 2005), Yersinia pestis (Nicas et al. 2005), Bacillus anthracis (Inglesby et al. 1999) and others.

In aerobiology, dose-response curves are useful for describing the probability (y-axis) that a specific dose (x-axis) of an airborne pathogen will produce infection in a susceptible host (Douwes et al. 2007; Pillai and Ricke 2002). Under experimental conditions, dose-response curves can be derived by individually exposing susceptible animal hosts to a known quantity of pathogen and then monitoring each animal for evidence of infection under conditions that preclude the possibility of infection from all other sources (French et al. 2002; Hermann et al. 2009). The proportion of individuals that become infected at each dose provides the raw data upon which the dose-response curve is based.

A variety of statistical techniques may be used to analyze the dose-response relationship (Hubert 1977), with the ID$_{50}$, the dose required to infect 50% of the population, being the most useful summary statistic of the dose-response for any defined pathogen-host system (Spouge 1992; Ward and Akin 1984). A standard dose-response curve is defined by four parameters: the baseline (bottom), the maximum response (top), the slope of the curve, and the mid-point of the curve (Motulsky and Christopoulos 2003), but the exact parameters of a dose-response curve depend on the strain or isolate of the pathogen (Ward and Akin 1984), the host species (Thurston-Enriquez 2003), and specific host factors, such as age and immune status (Jani et al. 2008).

In the research setting, host- and pathogen-specific factors that affect the dose-response curve can be accounted for by careful experimental design. A larger challenge is the requirement to determine the dose of infectious pathogen to which the host was exposed. Estimation of
exposure dose requires measurements on the total volume of air respired by the host and the concentration of viable airborne pathogen, e.g., liters of air respired by the susceptible host × pathogen concentration per liter of air = exposure dose. In domestic animals, total respired air can be measured using appropriate spirometric instrumentation (Hermann et al. 2009).

Estimates of the concentration of infectious airborne pathogen are often more difficult to achieve. By definition, quantification of viable airborne pathogens is based on techniques that require the microorganism to replicate in culture. Culture-based methods are less analytically sensitive than contemporary molecular techniques, e.g., polymerase chain reaction (PCR), but molecular assays cannot substitute because they do not differentiate between infectious and non-infectious microorganisms (Douwes et al. 2007; Ward and Akin 1984). If the pathogen is not highly transmissible, i.e., if transmission requires a large exposure dose, the determination of the dose-response curve may be achieved despite the requirement to quantify infectious microorganisms in culture. Not infrequently, airborne pathogens are transmissible at concentrations below the threshold of quantification by culture. Under these circumstances, the exposure dose, and hence the probability of transmission, is incalculable (Gillespie et al. 1996; Pallai and Ricke, 2003). In this paper we present an engineering approach to the calculation of exposure dose at microbiologically unquantifiable level.

The continuous-stirred tank reactor (CSTR) is a vessel characterized by steady-state and uniform internal conditions due to mixing (Hill 1997). The reactions and processes occurring within the CSTR may be well-defined for the conditions of the vessel. For example, the concentration of a target within a CSTR can be predicted at any time (t) using the equation:
\[ C_t = C_{in}(1 - e^{-t(Q/V)}) = C = C_{in}(1 - e^{-x}) \]

Where:
- \( C_t \) = target concentration at time = \( t \)
- \( C_{in} \) = target input concentration at time = 0
- \( t \) = time
- \( Q \) = flow rate (incoming rate = outgoing rate)
- \( V \) = volume of the CSTR
- \( e \) = the base of natural logarithm (Euler's number)
- \( x \) = one complete exchange of the volume of the CSTR

Table 1. Target concentration as a function of the number of complete exchanges of a continuous-stirred tank reactor (CSTR)

<table>
<thead>
<tr>
<th>Exchange ((x))</th>
<th>( C_t/C_{in} )</th>
<th>Target retained in the CSTR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( 1 - e^{-1} )</td>
<td>37.0</td>
</tr>
<tr>
<td>2</td>
<td>( 1 - e^{-2} )</td>
<td>14.0</td>
</tr>
<tr>
<td>3</td>
<td>( 1 - e^{-3} )</td>
<td>5.0</td>
</tr>
<tr>
<td>4</td>
<td>( 1 - e^{-4} )</td>
<td>1.8</td>
</tr>
<tr>
<td>5</td>
<td>( 1 - e^{-5} )</td>
<td>0.7</td>
</tr>
</tbody>
</table>

\(^1\) \( x \) = one complete exchange of the volume of the CSTR

CSTRs are widely used in a variety of industrial, chemical, and biological applications, including bioreactors, fermentation vessels, and wastewater treatment. Goldberg et al. (1958) introduced the use of a continuously rotating drum ("dynamic aerosol toroid") to experimental aerobiology as a method to maintain and study infectious particles suspended in aerosols over time. The rotating dynamic aerosol toroid (DAT) housed in an environment that preserves the pathogen's infectivity, e.g., held at temperatures below freezing, is a CSTR. As such, the concentrations of the airborne components within the DAT can be predicted at time \( t \) if the exchange volumes are known. This paper provides experimental evidence to support this concept and discusses the application of this approach to the problem of estimating the concentration of airborne pathogens at microbiologically unquantifiable levels.
2.0 MATERIALS AND METHODS

2.1 Experimental design
The objective of this experiment was to determine whether the CSTR-derived calculations could accurately predict the concentration of an airborne target in a DAT given known exchange volumes. As a surrogate for an airborne microorganism, a fluorescent dye (rhodamine B) was aerosolized into a 400 liter DAT held at -4°C. In 10 replicates, 12 air samples (200 liter each) were collected and the fluorescence measured. These data were test for: (1) a linear relationship between the concentration of rhodamine B removed \( \log_{10} M_{c, \text{out}(t)} \) and volume of aerosol removed \( V_{\text{extracted}} \) and (2) a significant difference between the theoretical and the observed rhodamine B regression lines.

2.2 Experimental procedures
A stainless steel 400 liter DAT was constructed based on the description provided by Goldberg et al. (1958). For temperature control, the DAT was housed in a custom-built refrigeration unit (Carroll Coolers, Inc., Carroll, IA) maintained at -4°C. During operation, the DAT rotated at 4 RPM (Brother International Gearmotors, Bridgewater, NJ). Three HEPA-filters (Fisher Scientific, Hampton, NH) were fixed to ports on the periphery of the DAT to allow for pressure equilibration during nebulization and impingement. The entire system was disassembled and cleaned between each of the 10 replicates.

In each replicate, approximately 50 ml of a 1X phosphate-buffered saline (PBS) (Thermo Scientific, Rockford, IL) solution containing 0.08% v/v rhodamine B (Sigma Chemical Co., St. Louis, MO.) and 0.1% v/v Antifoam A Emulsion (Sigma Chemical Co., St. Louis, MO.) was nebulized into the DAT using a 24-jet Collison nebulizer (BGI Inc., Waltham, MA) operating at 40 PSI. According to the manufacturer's specifications, these parameters aerosolized the solution at a rate of 1.1 ml per minute and produced particles 1.9 μm in diameter. After nebulization and prior to sampling, the cloud was allowed to equilibrate within the DAT for 60 minutes. This allowed for complete mixing, sedimentation, and thermal equilibration of aerosolized rhodamine B.
Air samples were collected using sterile AGI-30 glass impingers (Ace Glass, Vineland, NJ) containing 20 ml of sterile 1X PBS (Thermo Scientific) as collection fluid. Impingers were operated at a constant flow rate of 12.5 liters per minute using oil-less pumps (Fisher Scientific, Hampton, NH). Pump performance was monitored using a vacuum pressure gauge (Cato Western Inc., Tucson, AZ). Twelve 200 liter air samples were taken in succession, i.e., 6 complete evacuations of the DAT over a period of approximately 3.5 hours. All samples were maintained on ice until sampling was completed. Thereafter, a 1.5 ml aliquot of each sample was dispensed into a disposable ultraviolet transmissible cuvet (Fisher Scientific, Pittsburg, PA), allowed to warm to 20°C, and the amount of rhodamine B dye in each sample measured using a fluorometer (Turner BioSystems Inc., Sunnyvale, CA) equipped with a green optical kit (Turner BioSystems Inc.). Results were expressed as raw fluorescence units. Prior to each replicate, the fluorometer was evaluated using a rhodamine B solid standard (Turner BioSystems Inc., Sunnyvale, CA).

2.3 Analysis
To predict the concentration of an airborne target (e.g., rhodamine B) within a CSTR as samples are drawn from the drum and replaced with filtered inlet air, a mass balance equation of the target's concentration in the drum can be written as:

\[
\frac{d(M_{c,\text{drum}}m_a)}{dt} = m_a \left(M_{c,\text{in}} - M_{c,\text{out}}\right)
\]

Where:

- \(M_{c,\text{drum}}\) = mass fraction of the target inside drum, kg\(_{\text{target}}/kg_a\)
- \(m_a\) = mass of air inside drum, kg\(_a\)
- \(t\) = time, s
- \(\dot{m}_a\) = mass flow rate of air through drum, kg\(_a/s\)
- \(M_{c,\text{in}}\) = mass fraction of target entering drum, kg\(_{\text{target}}/kg_a\)
- \(M_{c,\text{out}}\) = mass fraction of target leaving drum, kg\(_{\text{target}}/kg_a\)
Based on the work of Goldberg et al. (1958), a DAT is a well-mixed vessel. Therefore, the mass fraction of target leaving the drum is representative of the mass fraction inside the drum and can be stated as:

\[
M_{c,drum} = M_{c,out}
\]

and equation (1) can be re-written as:

\[
\frac{d(M_{c,out}M_a)}{dt} = \dot{m}_a \{M_{c,in} - M_{c,out}\}
\]

Integrating equation (3) results in the common form of a perfectly mixed, but dynamically changing, mass fraction starting from a known initial mass fraction as:

\[
M_{c,out}(t) = M_{c,out}(t = 0)e^{-(t \dot{m}_a / m_a)}
\]

Equation (4) states that the mass fraction of target in a perfectly mixed drum at any time (t) \(M_{c,out}(t)\) is a function of the initial concentration inside the drum \(M_{c,out}(t = 0)\) and the exponential decay characterized by the mass of air inside the drum \(m_a\) and the mass flow rate of air through the drum \(\dot{m}_a\). Assuming constant air density, equation (4) can be further described by:

\[
M_{c,out}(t) = M_{c,out}(t = 0)e^{-\left(V_{extracted} / V_{drum}\right)}
\]

The starting point for determination of the concentration of the target at time (t) is the initial mass fraction within the drum \(M_{c,out}(t = 0)\). Determination of target concentration requires sampling the air and subsequent sample analysis. This extraction process results in an interruption of the initial mass fraction of the target.

Equation (5) can be used to back-calculate the initial target mass fraction \(t = 0\) within the drum from the initial extracted sample \(t = 1\). Thus, after the first sample extraction, some
known amount of drum air has been extracted \( V_{\text{extracted}} \) resulting in a mass fraction of \( M_{c,\text{out}}(t_1) \) providing an estimation of the initial mass fraction determined as:

\[
M_{c,\text{out}}(t = 0) = \frac{M_{c,\text{out}}(t_1)}{e^{-(V_{\text{extracted}},t_1/V_{\text{drum}})}}
\]

Equation (6) represents the initial mass fraction inside the drum. Since the drum behaves as a well-mixed vessel, all subsequent sample extractions and the resulting mass fractions will obey the mixing model as given in equation (5) using the estimate for the initial mass fraction given in equation (6). The final relationship becomes;

\[
M_{c,\text{out}}(t) = \frac{M_{c,\text{out}}(t_1)}{e^{-(V_{\text{extracted}},t_1/V_{\text{drum}})}} e^{-(V_{\text{extracted}}/V_{\text{drum}})}
\]

Where:

- \( M_{c,\text{out}}(t_1) \) = mass fraction from the first sampled extraction, kg\text{target}/kg\text{a}
- \( V_{\text{extracted}},t_1 \) = volume of drum air extracted for the first sample, liters
- \( V_{\text{drum}} \) = fixed volume of the drum, liters

Converting Equation 5 to \( \log_{10} \) format, the mass fraction of the DAT can be mathematically represented as:

\[
\log_{10} M_{c,\text{out}}(t) = \log_{10} M_{c,\text{out}}(t=0) - (\log_{10} e/V_{\text{drum}}) V_{\text{extracted}}
\]

Where:

- \( M_{c,\text{out}}(t=0) \) = concentration of rhodamine B at \( t = 0 \)
- \( M_{c,\text{out}}(t) \) = rhodamine B concentration at current time
- \( V_{\text{extracted}} \) = the running total of the volume removed
- \( V_{\text{drum}} \) = the total volume in container
- \( e \) = the base of natural logarithm (Euler's number).
This mathematical representation contained two assumptions:

1. There is a linear relationship between \( (\log_{10} M_{c,\text{out}}(t)) \) and \( V_{\text{extracted}} \):

2. The slope of the rhodamine B regression line was equal to \(-\left(\log_{10} e / V_{\text{drum}}\right)\).

If both of these assumptions are true, then, \( \log_{10} M_{c,\text{out}}(t) \) is a linear function of \( V_{\text{extracted}} \) with intercept \( \log_{10} M_{c,\text{out}}(t=0) \) and slope \(-\left(\log_{10} e / V_{\text{drum}}\right)\). Thus, the linear relationship between \( \log_{10} M_{c,\text{out}}(t) \) and \( V_{\text{extracted}} \) may be used to estimate the concentration of rhodamine B at any time along the regression line. To validate Assumption (a), the concentration of rhodamine B (\(\log_{10}\)) in sequential air samples collected in each of 9 replicates was analyzed using a simple linear regression model using the REG procedure in SAS® Version 9.2 (SAS® Institute Inc., Cary, NC) and the coefficient of determination (\(R^2\)) was calculated. To validate Assumption (b), the hypothesis that the average slope was equal to the theoretical slope \(-\left(\log_{10} e / V_{\text{drum}}\right)\) was tested using Students T.

### 3.0 RESULTS

A total of 10 replicates were attempted. One replicate (number 4) failed because of technical problems experienced during the run. For the remaining 9 runs, least square estimates of the intercept and slope as well as the coefficient of determination (\(R^2\)) were calculated for each regression line (Table 2). \(R^2\) described the proportion of response variation explained for by the linear model and ranges from 0 to 1, with a large \(R^2\) value indicative of a good fit of the linear model. The mean \(R^2\) for the nine replicates was 0.93 with a standard deviation of 0.07. Overall, the linear regression line explained 93% of the variation in \(\log_{10}\) transformed rhodamine B data. The average slope of the 9 runs was not significantly different (p=0.1593) from the theoretical slope (-0.0011). Therefore, these data support the conclusion that the linear relationship between \( \log_{10} M_{c,\text{out}}(t) \) and \( V_{\text{extracted}} \) may be used to estimate the concentration of an airborne target in a DAT given known exchange volumes.
Table 2. Parameters describing the linear relationship between the concentration of airborne rhodamine B and the volume of air extracted from a dynamic aerosol toroid

<table>
<thead>
<tr>
<th>Replicate</th>
<th>$R^2$</th>
<th>Slope</th>
<th>Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.96</td>
<td>-0.0013</td>
<td>3.47</td>
</tr>
<tr>
<td>2</td>
<td>0.83</td>
<td>-0.0021</td>
<td>3.40</td>
</tr>
<tr>
<td>3</td>
<td>0.96</td>
<td>-0.0030</td>
<td>3.16</td>
</tr>
<tr>
<td>5</td>
<td>0.94</td>
<td>-0.0007</td>
<td>3.20</td>
</tr>
<tr>
<td>6</td>
<td>0.95</td>
<td>-0.0011</td>
<td>4.07</td>
</tr>
<tr>
<td>7</td>
<td>0.80</td>
<td>-0.0007</td>
<td>2.56</td>
</tr>
<tr>
<td>8</td>
<td>0.97</td>
<td>-0.0012</td>
<td>4.08</td>
</tr>
<tr>
<td>9</td>
<td>0.99</td>
<td>-0.0013</td>
<td>4.72</td>
</tr>
<tr>
<td>10</td>
<td>0.98</td>
<td>-0.0016</td>
<td>4.70</td>
</tr>
</tbody>
</table>

4.0 DISCUSSION AND CONCLUSIONS

Successful airborne transmission occurs in three basic steps: (1) aerosolization of the infectious agent; (2) environmentally-dependent movement, dilution, and inactivation of airborne infectious particles; and (3) contact, entry, and replication within a susceptible host (Stärk 1999). From the perspective of prevention and control, the goal is to understand and model the transmission of airborne pathogens in order to design effective counter-measures. Both macro- and micro-level approaches are useful in meeting this objective. That is, field data collected over the course of an outbreak may be useful for modeling the airborne spread of a pathogen within a population (Keeling et al. 2001). Likewise, the basic steps and their components may be evaluated independently under controlled conditions to understand the contribution of each to the process of transmission (Hermann et al. 2009). That is: (1) quantify the rate at which the pathogen is excreted into the environment; (2) measure the rate of inactivation of the airborne infectious pathogen under specific environmental conditions; and (3) estimate the likelihood that exposure to a specific dose of the airborne infectious pathogen will produce a response (infection) in an individual host.
This study addressed the third step in this process and, in particular, the specific problem of deriving dose-response curves under experimental conditions in which transmission occurs at concentrations below the threshold of quantification for culture-based methods. In this experiment, a tracer was used to model the behavior of an aerosolized pathogen in a rotating DAT. Tracers, e.g., uranine, rhodamine B, and *Bacillus subtilis* spores, have been used extensively in experimental aerobiology (Verreault *et al.* 2008). Songer (1967) aerosolized rhodamine B dye simultaneously with virus (Newcastle disease virus, infectious bovine rhinotracheitis virus, vesicular stomatitis virus, T3 bacteriophage) to track the physical loss of airborne virus within a DAT. In an experiment of similar design, Hermann *et al.* 2007 found no significant difference between the slopes of rhodamine B dye and porcine reproductive and respiratory syndrome virus RNA detected by quantitative PCR, i.e., the concentrations of rhodamine B and viral RNA declined in the DAT at the same rate. Under the conditions of this experiment, the fact that the theoretical line and the experimental line were not significantly different provided evidence that physical loss did affect the outcome of the tracer values. Thus, rhodamine B concentration has been shown to reflect target pathogen concentrations under conditions similar to those reported here.

The physical parameters and experimental conditions of this study merit discussion. This experiment was conducted in a 400 liter DAT rotated at 4 RPM. However, a variety of DAT sizes and rotation speeds are reported in the literature, e.g., 140 liter (Songer 1967), 1000 liter (Adams *et al.* 1982), and 2,500 liter (Ehrlich 1964). A review of the literature found no evaluation of the effect of DAT dimensions, volume, and rate of rotation on the behavior of suspended particles. Therefore, it would be of value to confirm the results reported here using the described methodology.

In this experiment, the environmental conditions were designed to preserve target pathogen infectivity. In particular, the -4°C temperature at which the DAT was maintained would be expected to preserve the infectivity of a target pathogen indefinitely. At temperatures above freezing, the slope of the airborne pathogen would diverge from the slope of the rhodamine B. Therefore, the inactivation of the target pathogen over time would need to be accounted
for in the estimation of airborne pathogen concentration. This is not an insignificant consideration because the rate of airborne pathogen concentration inactivation is affected by isolate (Kelling et al. 2001; Stärk 1999), the suspension medium (Benbough 1971), Ehlrich et al., 1964), temperature (Elazhary and Derbyshire 1979), relative humidity (Hermann et al. 2007). Therefore, it is preferable to avoid this complication by maintaining the DAT at temperatures below 0°C.

In dose-response studies, the CSTR model solves the problem of estimating the exposure dose when the concentration of airborne pathogens at microbiologically unquantifiable levels. Specifically, the linear relationship between \( \log_{10} M_{c,\text{out}}(t) \) and \( V_{\text{extracted}} \) may be used to estimate the concentration of a target at any point along the regression line. Thus, \( \log_{10} M_{c,\text{out}}(t) \) is a linear function of \( V_{\text{extracted}} \) with intercept \( \log_{10} M_{c,\text{out}}(t=0) \) and slope \( -\left( \log_{10} e/V_{\text{drum}} \right) \). Therefore, under conditions similar to those reported here, the CSTR model solves the problem of estimating the exposure dose when the concentration of airborne pathogens is at microbiologically unquantifiable levels.

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CHAPTER 3. MEDIAN INFECTIOUS DOSE (ID$_{50}$) OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS ISOLATE MN-184 VIA AEROSOL EXPOSURE

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ABSTRACT
The median infectious dose (ID$_{50}$) of porcine reproductive and respiratory syndrome (PRRS) virus isolate MN-184 was determined for aerosol exposure. In 7 replicates, 3-week old pigs (n = 58) respired 10 liters of airborne PRRS virus from a dynamic aerosol toroid (DAT) maintained at -4°C. Thereafter, pigs were housed in isolation and monitored for evidence of infection. Infection occurred at virus concentrations too low to quantify by microinfectivity assays. Therefore, exposure dose was determined using two indirect methods ("calculated" and "theoretical"). "Calculated" virus dose was derived from the concentration of rhodamine B monitored over the exposure sequence. "Theoretical" virus dose was based on the continuous stirred-tank reactor model. The estimation of ID$_{50}$ was modeled on the proportion of pigs that became infected using the probit and logit link functions for both "calculated" and "theoretical" exposure doses. Based on "calculated" doses, the probit and logit ID$_{50}$ estimates were $1 \times 10^{-0.13}$ TCID$_{50}$ and $1 \times 10^{-0.14}$ TCID$_{50}$, respectively. Based on "theoretical" doses, the probit and logit ID$_{50}$ estimates were $1 \times 10^{0.26}$ TCID$_{50}$ and $1 \times 10^{0.24}$ TCID$_{50}$, respectively. For each point estimate, the 95% confidence interval included the other three point estimates. The results indicated that MN-184 was far more infectious than PRRS virus isolate VR-2332, the only other PRRS virus isolate for which ID$_{50}$ has been estimated for airborne exposure. Since aerosol ID$_{50}$ estimates are available for only these two isolates, it is uncertain whether one or both of these isolates represent the normal range of PRRS virus infectivity by this route.
1.0 INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) virus was first isolated in 1991 in the Netherlands and shortly thereafter in the United States (Collins et al., 1992; Collins, 1991; Terpstra et al., 1991; Wensvoort et al., 1991). The PRRS virus genome is polyadenylated, single-stranded, non-segmented, positive-sense RNA (Zimmerman et al., 2006). Because it is an RNA virus, PRRS viruses are recognized as genetically and antigenically highly variable (Meulenberg et al., 1993; Meulenberg, 2000; Stadejeck et. al., 2006; Yoon et al., 1999). Understanding this variability in the context of immunity, clinical expression, and pathology has been a major area of research focus (Andreyev et al., 2000; Christopher-Hennings, 2000; Corradi et al., 2005; Halbur et al., 1996a; Halbur et al., 1996b; Halbur et al., 2002; Meier et al., 2004; Meulenberg, 2000; Murtaugh, et al., 1995; Murtaugh et al., 2002; Opriessnig et al., 2002; Stadejek et al., 2006). In this paper we consider isolate variability in terms of infectivity.

In June 2001, commercial swine herds in southern Minnesota USA reported outbreaks of abortion (1 to 45%), pre-weaning mortality (28 to 55%), nursery mortality (8 to 35%) and finisher mortality (7 to 14%) associated with PRRS virus infections (J Torrison, personal communication). Among the PRRS viruses recovered from these outbreaks was an isolate found to possess a previously unrecognized restriction fragment length polymorphism pattern (RFLP 1-8-4), hence designated MN-184 (Cho et al., 2006; Faaberg et al., 2006). Genetic analyses showed that MN-184 shared approximately 87% and 59% ORF5 nucleotide identity with VR-2332 and Lelystad viruses, respectively. It was also determined that MN-184 possessed the shortest PRRS virus genome sequenced to date (Han et al., 2006). That is, the MN-184 genome consisted of 15019 nucleotides versus 15,111 nucleotides for the European prototype, Lelystad virus (GenBank M96262) and 15,182 nucleotides for the North American prototype, VR-2332 (GenBank EF536003) (Dee et al., 2010; Halburg et al., 1996b). In large part this was due to three deletions in the nsp2 region of ORF1a (replicase polyprotein) that resulted in the removal of 131 amino acids (Faaberg et al., 2006; Han et al., 2006; Han et al., 2007).
Initial clinical signs in the field and subsequent controlled studies led to the classification of MN-184 as a highly pathogenic PRRS virus isolate (Cano et al., 2007; Cho et al., 2007). In a direct comparison of five field isolates (MN-184, 171198-6, JA 142, SDSU 73, VR-2332), and three laboratory-attenuated viruses it was found that MN-184 induced the highest serum virus titers on day post inoculation (DPI) 7, 28, and 35 (Johnson et al., 2004). Likewise, MN-184-inoculated pigs had the highest rate of mortality (50%) over the course of the study and the lowest mean body weight among all PRRS virus-challenged groups at DPI 49.

The biodynamics of MN-184 have been describe in detail (Charerntantanakul et al., 2006; Cho et al., 2006; Cho et al., 2007; Dee et al., 2004; Dee et al., 2009; Otake et al., 2010). Cho et al. (2006) compared shedding patterns in pigs infected with isolates MN-30100 and MN-184. Detectable aerosol shedding of MN-184 was significantly more frequent than MN-30100, although virus concentrations in respiratory samples and nasal swabs did not differ (Cho et al., 2006, 2007). Subsequently, Cho et al. (2006) reported successful aerosol transmission of MN184 (4 of 10 attempts), but not isolate MN-30100 (0 of 10 attempts) under experimental conditions in which inoculated and recipient pigs were connected by a 1.3 meter duct. Air samples from chambers housing pigs inoculated with MN-184 were positive for virus (3 of 5 samples) by quantitative RT-PCR, whereas no airborne virus was detected (0 of 5 samples) in air samples from chambers housing pigs inoculated with MN-30100. These results were in agreement with earlier work reporting failure to achieve aerosol transmission using PRRS virus isolate MN-30100 (Mortensen et al., 2002). Based on these comparative studies, it has been postulated that the transmissibility of PRRS virus in aerosols differs among isolates (Cho et al., 2006, 2007; Murtaugh et al., 2002).

Methods for quantitative evaluation of PRRS virus transmissibility have been described (Hermann et al., 2005; Hermann et al., 2009). In brief, virus is aerosolized into a reservoir; animals respire a specific dose of airborne virus, and are then monitored to determine if the exposure resulted in infection. Whether exposure to a specific dose of airborne virus produces infection is a probability function described by the dose-response relationship between the pathogen and the host. The median infectious dose (ID$_{50}$), the dose at which
50% of those exposed become infected, is a useful summary statistic of the dose-response curve. That is, ID$_{50}$ is a measure of the infectivity of the specific pathogen that provides for the comparison infectivity among different pathogens and/or isolates. Previously, the ID$_{50}$ of airborne PRRS virus isolate VR-2332 was determined (Han et al., 2007). The objective of this study was to estimate the ID$_{50}$ of the PRRS virus isolate MN-184 via aerosol exposure and compare to the earlier ID$_{50}$ estimate for isolate VR-2332.

2.0 MATERIAL AND METHODS

2.1 Institutional compliance
The experiment and all procedures were approved by the Iowa State University Institutional Biosafety Committee (#07-I-029-A) and the Institutional Animal Care and Use Committee (#9-07-6429-S). Animals were housed and cared for in compliance with the requirements provided in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science Societies 1999).

2.2 Experimental design
The study was conducted in 10 replicates, with pigs randomly assigned to exposure dose. To conduct the experiment, 8 or 9 pigs per replicate were sequentially exposed to successively lower doses of airborne PRRS virus (Figure 1). Decreasing aerosolized viral exposure concentrations were achieved by removing 190 liters of air between each pig. Air samples collected before and after each pig were used to estimate the exposure dose. Serum samples collected from each pig 5 and 10 days post-exposure (DPE) were tested for the presence of PRRS virus to determine whether exposure resulted in infection. The dose-response curve for exposure to airborne PRRS virus was derived from the proportion of pigs infected by dose.

In each replicate, one pig (negative control) was used to validate biosecurity procedures, i.e., was present in the laboratory during the aerosol exposure treatments, was housed in the same room as pigs exposed to PRRS virus aerosols, and was monitored throughout the observation period. A second pig (positive control) was inoculated intramuscularly with 10 ml of fluid
from the final air sample in each run to verify that infectious PRRS virus was present in the air source to which pigs were exposed throughout the experiment. Failure to detect infectious virus in the final air sample by bioassay invalidated the entire replicate.

2.3 Animal care and housing

Pigs were received at approximately 3 weeks of age from a PRRS virus-negative herd. Throughout the experiment, pigs were fed a commercial pelleted diet (Kent Feeds Inc., Muscatine, IA) that met or exceeded the age-appropriate nutritional requirements for swine set by the National Research Council, (1998). To verify their PRRS virus-negative status, pigs were tested on -4 and 0 DPE for PRRS virus by virus isolation and anti-PRRS virus serum antibodies by ELISA (HerdChek® PRRS 2XR, IDEXX Laboratories, Inc., Portland, ME, USA).

Animals were individually housed in hepa-filtered (Flanders Filters, Inc., Washington, NC, USA) isolation units (Barrier Systems, Inc., Toms River, NJ, USA). Isolation units were equipped with air, feed, and waste handling systems that maintained a biosecure environment and prevented transmission of PRRS virus between pigs. Between replicates, the isolation units were cleaned and disinfected with chlorhexidine diacetate (#Nolvasan1 Solution; Fort Dodge Laboratories, Fort Dodge, IA, USA) and then left empty for at least 48 h with the heating system set at 32°C and the hepa-filter air system in operation.

2.4 Virus propagation

A type 2 PRRS virus isolate, MN-184 (kindly provided by Dr. Scott Dee, University of Minnesota, MN, USA) was propagated on MARC-145 cells, a clone of the African monkey kidney cell line MA-104 (Kim et al., 1993). Cells were grown in 162 cm² flasks (Corning, Corning, NY, USA) using Dulbecco’s Modified Eagles Medium (DMEM) (Mediatech, Inc., Manassas, VA, USA) supplemented with 0.25 μg/ml Amphotericin B (Sigma Chemical Co., St. Louis, MO, USA), 50 μg/ml gentamicin (Sigma), 0.5 molar L-glutamine (Fisher Scientific, Hampton, NH, USA), 300 international units (IU) per ml penicillin (Sigma), 300 μg/ml streptomycin (Sigma), 1.0% nonessential amino acids (HyClone, Logan, UT, USA),
25 mM HEPES buffer (Sigma), and 10.0% heat-inactivated fetal bovine serum (FBS) (Sigma, verified BVDV free). When cells were confluent (72 to 84 hrs), the media was discarded and the flasks inoculated with 5 ml DMEM (without L-glutamine) containing PRRS virus isolate MN-184 at a titer of $1 \times 10^{3.5}$ median tissue culture infective dose (TCID$_{50}$) per ml. Flasks were placed on a rocking platform in a 37°C humidified 5% CO$_2$ incubator for 90 min, then 40 ml of modified DMEM growth medium with 4% FBS was added and the flasks returned to the incubator for 36 hrs. Cell culture supernatant was harvested by flask freeze-thaw and centrifugation (3000 x g for 20 min at 4°C). Virus stock was stored in 25 ml aliquots at -80°C.

2.5 Microinfectivity assays
Samples to be quantified for PRRS virus were serially 10-fold diluted (10$^{-0}$ to 10$^{-5}$) in DMEM growth medium without FBS and assayed in triplicate on confluent MARC-145 cells propagated in 96-well tissue culture plates (Corning). Growth medium was discarded from plates, 5 wells were inoculated with 100 $\mu$l of sample at each dilution, and the plates placed in a 37°C humidified 5% CO$_2$ incubator for 90 min. The inoculum was then discarded, 100 $\mu$l per well of growth medium containing 4% FBS was added to each well, and the plates incubated at 37°C in a humidified 5% CO$_2$ incubator for 24 hrs. Following incubation, cells were fixed with cold 80% acetone/water solution and stained with a fluorescein isothiocyanate-conjugated (FITC) monoclonal antibody specific for PRRS virus (Rural Technologies Inc., Brookings, SD, USA). Virus titers (TCID$_{50}$ per ml) and 95% confidence intervals were calculated based on the number of wells showing PRRS virus-specific fluorescence at each dilution using Spearman-Kärber and logit methods (Spouge, 1991).

2.6 Virus aerosolization, air sampling, and sample handling
Airborne PRRS virus for pig exposure was provided by nebulizing PRRS virus into a 400 liter stainless steel dynamic aerosol torrid (DAT) rotating at 4 RPM (Brother International Gearmotors, Bridgewater, NJ, USA). Virus infectivity was maintained by housing the entire apparatus within a custom-built refrigeration unit held at -4°C (Carroll Coolers, Inc., Carroll, IA, USA). To allow for pressure equilibration during nebulization, impingement, and pig
exposure, three HEPA-filters (Fisher Scientific, Hampton, NH, USA) were fixed to ports on the circumference of the DAT. The entire system was disassembled, cleaned, and disinfected between replicates. For each replicate, a solution consisting of virus stock (1 x 10^{7.7} TCID_{50} per ml), 0.08% v/v rhodamine B dye (Sigma Chemical Co.), and 0.1% v/v Antifoam A Emulsion (Sigma Chemical Co.) was aerosolized into the DAT using a 24-jet Collison nebulizer (BGI Inc., Waltham, MA, USA), operating at 40 PSI. According to the manufacturer, the solution was aerosolized at a rate of 1.1 ml per minute and produced particles 1.9 μm in diameter. The aerosol was allowed to mix and stabilize within the DAT for 60 minutes after nebulization.

Air samples were collected from the DAT over the course of each replicate to monitor the concentration of airborne PRRS virus and rhodamine B, i.e., 200 liter air samples (n = 4) were collected following the stabilization period and 190 liter samples were collected following each pig exposure. Air samples were collected using 20 ml of sterile 1X PBS in sterile AGI-30 glass impingers, (Ace Glass, Vineland, NJ, USA), operated at a constant flow rate of 12.5 liter per minute. Impinger collection bottles were placed on ice during the collection process to maintain virus viability. Following collection, bottles were maintained on ice for transport to the laboratory. Sample collection fluid was assayed for the concentration of PRRS virus (described above) and rhodamine B dye. Virus microinfectivity assays were performed immediately post sample collection, the concentration of rhodamine B dye was quantified using a fluorometer (Model - 9200-000, Turner BioSystems Inc., Sunnyvale, CA, USA) equipped with a green optical kit (Model - 9200-042, Turner BioSystems Inc.) and expressed as raw fluorescence units. Fluorometer performance was verified prior to each replicate using a rhodamine B solid standard (Turner BioSystems Inc.).

2.7 Animal exposure to virus aerosol
Pigs were removed from individual isolation units on day 0, bled, and anesthetized using a solution formulated by reconstituting Telazol (250 mg of tiletamine, 250 mg of zolazepam; Fort Dodge Animal Health, Fort Dodge, IA, USA) with 2.5 ml of xylazine (100 mg/ml; Lloyd Laboratories, Shenandoah, IA, USA) and 2.5 ml of ketamine (100 mg/ml; Fort Dodge
Animal Health). The solution was administered intravenously at a dose of 0.025 ml per kg of body weight.

For exposure to the virus aerosol, the entire anterior portion of the head, i.e., snout and mouth, of the anesthetized pig was fitted with a canine surgical mask (Model - 32393B1, SurgiVet, Waukasha, WI, USA). To prevent rebreathing of respired aerosol, a one-way valve (Model - BE-117, Instrumentation Industries, Inc., Bethel Park, PA, USA) was attached to the tubing inserted into the DAT containing aerosolized infectious PRRS virus. Each pig respired 10 liters of PRRS virus aerosol. The exposure time was recorded and the cumulative volume (liters) of air respired by the animal during the exposure period was measured using a pediatric spirometer (Model 8805, Boehringer Laboratories Inc., Norristown, PA, USA). The one-way valve, tubing and mask were replaced between animals (Figure 1).

2.8 Determination of infection status post-exposure
Serum samples were collected using a single-use blood collection system (Becton Dickinson, Franklin Lakes, NJ, USA). Blood samples were centrifuged at 3000 x g for 10 min, after which the serum was harvested and stored at -80°C. Samples were collected on DPE -4, 0, 5, and 10. Samples were tested for anti-PRRS virus antibodies using a commercial PRRS ELISA (IDEXX HerdChek® PRRS 2XR, IDEXX Laboratories, Westbrook, ME, USA) and by virus isolation. Virus isolation on serum samples was performed as described above by inoculating wells of a 96-well plate with undiluted serum. A positive result was indicated by a PRRS virus-specific fluorescence reaction.

2.9 Analysis
2.9.1 Determination of PRRS virus aerosol exposure dose
Determination of aerosol exposure dose required precise information on the concentration of airborne PRRS virus within the DAT. Impinger fluid concentration of PRRS virus (TCID\textsubscript{50}) in the first DAT aerosol sample was used to determine the initial virus (per liter) concentration of aerosol:
\[ T_a = \left( T_o \times V_f \right) / L_t \]

Where:
- \( T_a \) = PRRS virus TCID\(_{50}\) per liter of air within the DAT
- \( T_o \) = PRRS virus TCID\(_{50}\) per ml of impinger fluid
- \( V_f \) = Total volume of impinger fluid in milliters
- \( L_t \) = Liters of air impinged (sampled)

The concentration of PRRS virus in subsequent impinger samples was below the threshold of quantification by the microinfectivity assay. Therefore, two indirect methods were used to establish virus concentration per liter of air. For both methods, a key point is that the DAT was maintained below freezing (-4°C). That is, the indirect methods were valid because the frozen virus did not lose infectivity over the course of the exposure sequence.

### 2.9.1.1 Calculated virus concentration

Hermann et al. (2007) reported no statistically significant difference \((p = 0.99)\) in the slopes of rhodamine B and PRRS virus RNA concentrations in a study conducted in the DAT environment. These results affirmed that the slope of rhodamine B could be used to calculate airborne PRRS virus concentration within the DAT over the course of the experiment. Results based on this approach are hereafter referred to as "calculated" estimates.

### 2.9.1.2 Theoretical virus concentration

The second indirect method was based on the continuous stirred-tank reactor model (CSTR). Validation of this model in context of aerosols contained with a DAT is reported elsewhere (Cutler et al., 2010). In brief, a CSTR is a vessel characterized by steady-state and uniform internal conditions due to mixing (Hill, 1977). The application of this model to this experiment results in the following relationships:
(2) \[ C_{ri} = C_{ro} \times e^{(Vtr/Vt)} \]

Where:

- \( C_{ri} \) = Virus or rhodamine B concentration per liter of air for Time (t)
- \( C_{ro} \) = Virus or rhodamine B concentration at Time 0
- \( Vtr \) = Cumulative volume of air removed from the DAT
- \( Vt \) = Total volume of the DAT (400 liters)
- \( e \) = Base of natural logarithm (Euler’s number)

Log transformation of equation 2 results in a linear response that allowed for the prediction of virus or rhodamine B concentration:

(3) \[ \log_{10} C_{ri} = \log_{10} C_{ro} - (\log_{10}(e/Vt)) \times Vtr \]

Equation 3 described a linear relationship between the concentration of an airborne target and the volume of air removed from the DAT in which the concentration of the target is a function of the slope \(- (\log_{10}(e/Vt))\). Therefore, the level of the target (PRRS virus or rhodamine B) could be estimated for any time \( C_{ri} \) during the sampling sequence based on the initial virus concentration. Results based on this approach are hereafter referred to as "theoretical" estimates.

2.9.1.3 Pig virus aerosol exposure dose

The PRRS virus aerosol exposure dose for each animal was determined by multiplying the airborne PRRS virus concentration per liter of air at the specific exposure point by the number of liters the pig respired (10 liters). Two virus concentration estimates ("calculated" and "theoretical") were derived and used in the analysis.

2.9.2 Dose-response: Probability of infection by exposure dose

Each pig was considered an experimental unit because animals were treated individually and housed individually in HEPA-filtered units. The outcome was defined as a binary variable, i.e., aerosol exposure to a specific dose of PPRSV either produced infection or did not.
Among the 58 pigs exposed to aerosolized PRRS virus 34 pigs became infected.

The probability of infection after aerosol exposure was modeled on the proportion of pigs that became infected by dose. The generalized linear regression models (logit and probit link functions) were used to fit the binary response (infection) with the explanatory variable (exposure dose) using the PROBIT procedure of SAS® Version 9.1.3 Service Pack 4 of the SAS® System for Windows (SAS® Institute Inc., Cary, NC, USA). The logit model was represented as \( \ln \left( \frac{P}{1-P} \right) \), where \( P \) represents the probability that an individual chosen at random will become infected by a given dose “x”. \( P \) may be obtained from the logistic function:

\[
(4) \quad P = \left[ 1 + \exp(-\alpha - \beta x) \right]^{-1}, -\infty < x < \infty
\]

Logit \( P \) was estimated from the linear functions logit \( P = \alpha + \beta x \) (Govindarajulu, 2001).

In the probit model, \( P \) was obtained from the cumulative distribution function of the standard normal distribution (\( \Phi \)):

\[
(5) \quad P = (2\pi)^{-1/2} \int_{-\infty}^{\alpha+\beta} \exp \left( -\frac{u^2}{2} \right) du, -\infty < x < \infty
\]

Subsequently, probit \( P \) was estimated form the function probit \( P \) = normal deviate = \( \alpha + \beta x \) (Govindarajulu, 2001).

The median infective dose (ID$_{50}$) point estimate and 95% confidence intervals were estimated for each model and for both calculated and theoretical PRRS virus concentrations. The ID$_{50}$ estimates were expressed as TCID$_{50}$, i.e., the quantity of virus necessary to infect 50% of the pigs exposed to aerosolized PRRS virus isolate MN-184.
3.0 RESULTS

Serum samples from pigs collected on -4 and 0 DPE were negative for anti-PRRS virus antibodies using a commercial PRRS ELISA and by virus isolation indicating that animals had not had prior exposure to PRRS virus. Each replicate consisted of 8 or 9 pigs exposed to PRRS virus aerosol, one negative control pig, and one positive control pig. Among all pigs, the mean body weight on DPE 0 was 12.8 kg (range 10.2 to 17.5 kg). Among aerosol-exposed pigs, 10 liters of PRRS virus aerosol was respired in a mean time of 5 min 6 sec (range 2 min 21 sec to 7 min 42 sec). Following exposure, no mortality occurred during the monitoring period and it was not possible to determine whether pigs had become infected with PRRS virus on the basis of gross appearance. That is, no overt clinical signs were observed.

Of the 10 replicates, data from 3 replicates were excluded from the analysis. (1) One replicate was voided because the negative control became infected with PRRS virus, indicating a breach in protocol. (2) One replicate was voided because the positive control did not become infected, indicating that viable PRRS virus was not present throughout the exposure sequence. (3) One replicate was voided due to mechanical failure during the replicate resulting in improper air sampling. Thus, the statistical analysis was based on 7 successful replicates in which 34 of 58 (59%) pigs became infected by exposure to airborne PRRS virus.

Impinger fluid concentration of PRRS virus (TCID$_{50}$) in the first DAT aerosol sample ranged from $1 \times 10^{1.1}$ (95% CI: $10^{0.8}$, $10^{1.4}$) to $1 \times 10^{3.6}$ (95% CI: $10^{3.2}$, $10^{4.1}$) TCID$_{50}$ per ml. All subsequent analysis were based on these estimates. "Theoretical" and "calculated" ID$_{50}$ estimates derived for probit and logit models are given in Table 1 and shown in Figure 2.

4.0 DISCUSSION

Porcine reproductive and respiratory syndrome virus has long been recognized as highly infectious, i.e., Yoon et al. (1999) found that less than $1 \times 10^{1}$ TCID$_{50}$ was sufficient to infect pigs via intramuscular exposure with PRRSV virus isolate ISU-P. The purpose of present
study was to quantify the infectivity of a PRRS virus isolate administered to pigs via aerosol exposure by calculating the median infectious dose (ID$_{50}$). ID$_{50}$, the dose necessary to infect 50% of the exposed population, is derived from the dose-response curve describing the relationship between the level of exposure to a microorganism (dose), and the probability that the exposure will result in infection (response). For the same agent, the ID$_{50}$ may vary by the route of exposure, e.g., the ID$_{50}$ via oral exposure for PRRS virus isolate VR-2332 was estimated at $1 \times 10^{5.3}$ TCID$_{50}$ versus $1 \times 10^{4.0}$ TCID$_{50}$ for intranasal exposure (Hermann et al., 2005). As well, ID$_{50}$s may vary among isolates from the same species, e.g., Sellers and Gloster (2007) reported that infectivity differed both between foot-and-mouth disease virus (FMDV) types (O, A, C) and among isolates within types.

In the present study, the most conservative ID$_{50}$ estimate for aerosol exposure to isolate MN-184 was $1 \times 10^{0.26}$ TCID$_{50}$ (Table 1), with the 95% confidence intervals encompassing the other 3 point estimates. In contrast, Hermann et al. (2009), reported an ID$_{50}$ of $1 \times 10^{3.1}$ TCID$_{50}$ by aerosol exposure using isolate VR-2332. Both estimates were produced by the same laboratory working with pigs of approximately the same age and using the same experimental design, equipment, and protocols. For this reason, it may be concluded that both estimates are valid and the divergent estimates represent a true difference between the two PRRS virus isolates. Overall, the results raise the possibility of a distribution of ID$_{50}$s for the airborne route among PRRS virus isolates, a distribution for which we have only two ID$_{50}$ point estimates.

Early in the PRRS virus pandemic, the pattern of transmission was highly suggestive of airborne transmission (Goyal, 1993; Le Potier et al., 1997). This impression was strengthened by epidemiological studies suggesting that PRRS virus could be transmitted over distances of 1.25 m (Wills et al., 1997), 0.5 km (Le Potier et al., 1997), and up to 3 km (Mortensen et al., 2002). However, experimental work produced inconsistent reproduction of airborne transmission. For example, Torremorell et al. (1997) demonstrated transmission of PRRS virus isolate VR-2332 over a distance of one meter in one of 2 attempts, but were unable to detect PRRS virus in 125 liter air samples collected on each of 3 days. Otake et al.
(2002) reported transmission of PRRS virus isolate MN-30100 over a distance of 2.5 meters from infected animals to susceptible pigs sharing the same air space, but aerosols emitted from exhaust fans over distances of one to 30 meters did not transmit PRRS virus to sentinel pigs. The first exception to this pattern of poor aerosol transmissibility was a report of airborne transmission of PRRSV–EU across a distance of one meter distance in each of 3 attempts (Kristensen et al., 2004). The most comprehensive research on aerosol transmission has been done with PRRS virus isolate MN-184. Cho et al. (2007) reported that, in contrast to isolate MN-30100, only isolate MN-184 produced successful transmission to susceptible pigs and only MN-184 was detected in air samples. Using MN-184 infected animals as the source, Dee et al. (2010) successfully infected 14 of 26 replicates (10 pigs per replicate) at a distance of 120 meters.

Particularly under experimental conditions in which host infection and immune status can be established with certainty, it may be assumed differences in infectivity are primarily driven by viral genetics. As reviewed by Murtaugh et al. (2010), on-going genetic evolution of PRRS viruses is the result of mutation resulting from RNA polymerase infidelity and genomic recombination. It may be speculated that differences in infectivity among PRRS viruses result from genetic changes that reflect greater adaptation of the virus to its porcine host. Such adaptation has been shown to occur in other viruses. For example, Fry et al. (2005) reported that FMDV acquired the capacity to bind to heparin sulphate receptors under selection pressure in cell culture, rather than the targeted integrin receptors. PRRS virus isolate VR-2332, the only other isolate for which we have an ID_{50} estimate for aerosol exposure, was recovered in 1990 during the height of the North American PRRS virus epidemic and perhaps no more than 5 years after the virus was introduced in the continent (Benfield et al., 1992; Zimmerman et al., 1997). In contrast, isolate MN-184 was isolated in 2001 (Johnson et al., 2004). This additional decade of replication in pigs may have provided for further adaptation to the virus' porcine host that is reflected in its greater infectivity.

Data critical to interpreting the results of this study are missing. Specifically, it is not known whether the greater infectivity demonstrated for MN-184 by aerosol exposure is also
reflected in greater infectivity by oral, intranasal, or percutaneous exposures. Likewise, in terms of understanding the mechanisms underlying these biological differences, the paucity of ID$_{50}$ estimates for PRRS viruses precludes hypothesis testing.

5.0 CONCLUSION

The present work describes a standardized protocol for determining the ID$_{50}$ for viruses in aerosols. This methodology provided the basis for demonstrating differences among PRRS virus isolates in ID$_{50}$ by aerosol exposure. Questions remain to be answer regarding the range, distribution, and genetic basis of such differences. Likewise, the relationship between ID$_{50}$s by aerosol, oral, percutaneous, and intranasal exposures and the factors that drive virus infectivity remain to be established. Logically, modified live vaccine viruses should be evaluated and screened for infectivity in order to select for vaccines with a reduced potential for unintended transmission.

ACKNOWLEDGMENTS

This study was supported in part by Pork Checkoff funds distributed through the National Pork Board, P.O. Box 9114, Des Moines, IA 50306 and the PRRS CAP USDA NIFA Award 2008-55620-19132.

REFERENCES


Figure 1. Diagram of the experimental procedure for exposing pigs to aerosolized porcine reproductive and respiratory syndrome (PRRS) virus (From Hermann, et al., 2009 by permission of the publisher)

1. Modified refrigerator-freezer unit maintained at -4°C
2. Dynamic aerosol toroid (DAT) for holding virus aerosol in suspension
   A. Port for connection to DAT
   B. Collision nebulizer for generating PRRS virus aerosol
   C. Pediatric spirometer for measuring cumulative respired volume
   D. Hepa-filtered exhaust
   E. Canine anesthesia mask
   F. Impinger for collecting air samples
3. Temperature and DAT rotation controls
Figure 2. Probit and logit median infectious dose (ID₅₀) estimates of aerosolized infectious PRRS virus. [A = Probit ID₅₀ based on "theoretical" virus concentration; B = Probit ID₅₀ based on "calculated" virus concentration; C = Logit ID₅₀ based on theoretical virus concentrations; D = Logit ID₅₀ based on calculated virus concentration]
Table 1. Median infectious dose (ID50) point estimates and 95% confidence intervals expressed as median tissue culture infectious dose (TCID50)

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<th>ID_{50} 95% confidence interval</th>
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ABSTRACT

Aims: The objective of this study was to estimate UV$_{254}$ inactivation constants for four viral pathogens: influenza A virus, porcine respiratory and reproductive syndrome virus (PRRSV), bovine virus diarrhea virus (BVDV), and reovirus.

Methods and Results: Viruses in culture medium were exposed to one of 9 doses of UV$_{254}$ and then titrated for infectious virus. Analysis showed that virus inactivation by UV$_{254}$ was more accurately described by a two-stage inactivation model versus a standard one-stage inactivation model.

Conclusions: The results provided evidence for the existence of two heterogeneous virus subpopulations among the viruses tested, one highly susceptible to UV$_{254}$ inactivation and the other more resistant. Importantly, inactivation constants based on the one-stage inactivation model would have underestimated the UV$_{254}$ dose required for inactivation of these viruses under the conditions of the experiment.

Significance and Impact: To improve the accuracy of estimates, it is recommended that research involving inactivation of microorganisms evaluate inactivation kinetics using both one-stage and two-stage models. These results will be of interest to persons responsible for microbial agents under laboratory or field conditions.
INTRODUCTION

Ultraviolet (UV) radiation is the part of the electromagnetic spectrum that lays between the high energy X-rays (≤ 100 nm) and the visible spectrum (> 400 nm). UV\textsubscript{254} inactivation has been a proven method of disinfection since the 1930s (Brickner \textit{et al.} 2003). In the 1960s, it was shown that inactivation of microbes occurred through dimer formation by nucleic acids, with 260nm determined to be the UV wavelength most efficiently absorbed by genetic material (Kowalski \textit{et al.} 2001).

The efficacy of ultraviolet inactivation is dependent upon several factors, including the microbe’s susceptibility to UV\textsubscript{254}. Microbial susceptibility to UV\textsubscript{254}, generally termed the inactivation constant (\(k\)), is frequently modeled using the following:

\[
(1) \quad k = \frac{\log_{10}\left(\frac{N_t}{N_0}\right)}{D_{o s e t}}
\]

Where:

- \(k\) = the inactivation constant
- \(N_0\) = quantity of microbes at time zero
- \(N_t\) = quantity of microbes at UV\textsubscript{254} exposure time "t"
- \(D_{o s e t}\) = depth (centimeters) of the solution irradiated by UV\textsubscript{254}

Thus, the one-stage inactivation model (Equation 1) describes the one-hit model which, when \(\log_{10}\) transformed, is described by a linear relationship between the survival fraction of the microbial population and the UV\textsubscript{254} exposure dose (Goldberg \textit{et al.} 1958). This model long served as the primary method for describing microbial susceptibility to UV\textsubscript{254}. In 1964, Hiatt described the limitations of the model and proposed several alternate computational models to describe the inactivation of microbes (Hiatt 1964). More recently Kowalski \textit{et al.} (2001) described a two-stage inactivation model for UV\textsubscript{254} inactivation of viruses. The objective of the present study was to compare the one-stage and two-stage inactivation models and report the inactivation constants for two enveloped, (+)ssRNA viruses (porcine respiratory and reproductive syndrome virus, and bovine virus diarrhea virus), an enveloped (-)ssRNA virus
(influenza virus type A), and a non-enveloped dsRNA virus (reovirus) in a static environment. The viruses chosen include economically significant animal pathogens for which estimates of UV$_{254}$ inactivation are not available.

**MATERIALS AND METHODS**

**Experimental design**
Viruses were suspended in culture medium and exposed to specific doses of UV$_{254}$ by placing aliquots into wells of a modified 8-well plate (VWR, West Chester, PA, USA). Each plate included a positive control virus (BVDV, PRRSV, reovirus, or influenza) which was protected from UV$_{254}$ exposure by covering the well with aluminum foil. To reduce bias, the placement of each virus within the plate, the unexposed virus control for each plate, and the UV$_{254}$ dose for each plate was randomized. Following UV$_{254}$ exposure, the samples were harvested and stored at -80°C until titrated for infectious virus. Virus inactivation data were analyzed using both the one-stage inactivation and two-stage inactivation models (Goldberg *et al.* 1958; Kowalski *et al.* 2001).

**Selected viruses**

**Influenza virus** Influenza A/Swine/Iowa/73 (H1N1) (USDA: National Veterinary Service Laboratories, Ames, IA, USA) was propagated on Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection (ATCC), Manassas, VA, USA). Cells were grown in 162 cm$^2$ flasks (Corning, Corning, NY, USA) using Dulbecco’s Modified Eagles Medium (DMEM) (Mediatech Inc., Manassas, VA, USA) supplemented with 0.25 µg ml$^{-1}$ Amphotericin B (Sigma Chemical Co., St. Louis, MO, USA), 50 µg ml$^{-1}$ gentomycin (Sigma Chemical Co.), 0.5 molar L-glutamine (Gibco, Carlsbad, CA, USA), 300 international units (IU) ml$^{-1}$ penicillin (Sigma Chemical Co.), 300 µg ml$^{-1}$ streptomycin (Sigma Chemical Co.), 1.0% nonessential amino acids (HyClone, Logan, Utah, USA), 25 mmol L$^{-1}$ HEPES buffer (Sigma Chemical Co.), and 10.0% heat-inactivated fetal bovine serum (FBS) (Sigma Chemical Co., verified BVDV free). When cells were confluent, the media was discarded and the flasks rinsed with 15 ml of complete infecting medium (CIM): 500 ml DMEM growth medium, 7.8% bovine serum albumin (BSA) (Sigma Chemical Co.)
in place of 10% FBS, and 0.83 ml of trypsin working stock. Trypsin working stock was formulated at 100 mg of L-(tosylamido-2-phenyl) ethyl chloromethylketone (TPCK-treated) trypsin (Worthington Biochemical, Lakewood, NJ, USA) diluted into 100 ml of CIM. Flasks were inoculated with 5 ml of CIM containing influenza virus at a titer of $1.26 \times 10^6$ TCID$_{50}$ ml$^{-1}$ and placed on a rocking platform in a 37°C humidified 5% CO$_2$ incubator for 90 min, after which the inoculate was discarded and replaced with 50 ml of maintenance medium (MM): DMEM growth medium with 4% heat-inactivated FBS in place of 10% FBS. Flasks were incubated at 37°C in a humidified 5% CO$_2$ incubator and cells examined daily for cytopathic effect (CPE). When ≥75% CPE was observed, flasks were freeze-thawed and the cell lysate harvested. The harvested lysate was centrifuged at 10,000 x g for 20 minutes at 4°C, decanted, and the supernatant stored at -80°C.

**Porcine reproductive and respiratory syndrome virus** A type 2 PRRSV isolate MN-184 (provided by Dr. Scott Dee, University of Minnesota) was propagated in MARC-145 cells (Iowa State University, Veterinary Diagnostic Laboratory, Ames, IA, USA), confluent cells were inoculated with 5ml of MN-184 virus at a titer of $1 \times 10^{3.5}$ TCID$_{50}$ ml$^{-1}$. The flasks were placed on a rocking platform in a 37°C humidified 5% CO$_2$ incubator for 90 min, then 40 ml of DMEM growth medium with 4% heat-inactivated FBS in place of 10% FBS, was added and the flasks returned to the incubator for 36 hrs. Cell culture supernatant was harvested by flask freeze-thaw and centrifugation (10,000 x g for 20 minutes at 4°C). Virus stock was stored in 25 ml aliquots at -80°C.

**Bovine viral diarrhea virus** Singer strain bovine viral diarrhea virus (BVDV) type 2 (provided by Dr. J. Ridpath, USDA: NADC) was propagated on bovine turbinate cell line T7 (provided by Dr. J. Ridpath, USDA: NADC). The cells were grown in 162 cm$^2$ flasks using EME growth medium: EME medium (Sigma Chemical Co.) containing 0.25 μg ml$^{-1}$ Amphotericin B (Sigma Chemical Co.), 50 μg ml$^{-1}$ gentomycin (Sigma Chemical Co.), 300 IU ml$^{-1}$ penicillin (Sigma Chemical Co.), 300 μg ml$^{-1}$ streptomycin (Sigma Chemical Co.), and supplemented with 10% FBS (Sigma Chemical Co., verified BVDV free). When cells were 75% confluent, the media was discarded and the flasks inoculated with 5 ml EME
growth medium containing BVDV at a titer of $1.26 \times 10^6$ TCID$_{50}$ ml$^{-1}$ and placed on a rocking platform in a 37°C humidified 5% CO$_2$ incubator for 90 min. Thereafter, 40 ml of EME growth medium was added and the cells examined daily for cytopathic effect (CPE). When approximately 75% CPE was observed, cell culture supernatant was harvested by flask freeze-thaw and centrifugation (10,000 x g for 20 minutes at 4°C). Virus stock was stored in 25 ml aliquots at -80°C.

**Reovirus**  
Reovirus strain T3DC (provided by Dr. Cathy Miller, Iowa State University) was propagated in spinner culture-adapted murine fibroblasts (L929 cells) as described in Qin et al. (2009). Confluent L929 cells were incubated with a multiplicity of infection of 10 plaque forming units per cell in Joklik c-MEM (Sigma Chemical Co.) and stirred for 3 days at 35°C. Thereafter, the cells were pelleted, the supernatant discarded, and the pellet re-suspended in an equal volume of fresh media. The re-suspended cells were sonicated, as described by Nibert and Fields (1992), and then centrifuged at 3000 x g for 5 min. The supernatant was adjusted to a virus concentration of $1.7 \times 10^9$ PFU ml$^{-1}$ with Joklik c-MEM media and stored at 4°C until used.

**Virus titration**  
Samples were assayed for infectious virus following the procedures described below. To avoid bias, sample aliquots were assayed blind and in random order. Virus titers were calculated using the Spearman-Kärber method and expressed as the median tissue culture infective dose (TCID$_{50}$) ml$^{-1}$ (Huber 1992).

**Influenza virus microinfectivity and hemagglutination assays**  
Swine testicle (ST) cells (ATCC CRL-1746) were propagated in 96-well tissue culture plates (Costar, Corning, NY, USA) using MEM growth medium: (MEM) minimum essential medium eagle (Sigma Chemical Co.) containing 0.25 μg ml$^{-1}$ Amphotericin B (Sigma Chemical Co.) 50 μg ml$^{-1}$ gentomycin (Sigma Chemical Co.), 2mmol L$^{-1}$ L-glutamine (Sigma Chemical Co.), 100 IU ml$^{-1}$ penicillin (Sigma Chemical Co.), 100 μg ml$^{-1}$ streptomycin (Sigma Chemical Co.), and supplemented with 10% heat inactivated fetal bovine serum (Atlas, Fort Collins, CO, USA).
Duplicate, samples were serially 10-fold diluted (10^{-1} to 10^{-6}) in MEM growth medium with 2% heat-inactivated FBS in place of 10% FBS. Test and control sample serial dilutions were plated at 100 µl per well onto confluent cells. Plates were incubated in a 37°C humidified 5% CO₂ incubator and observed daily, when ≥50% CPE was observed, or at the end of the 7 day incubation, the plates were frozen at -80°C.

All samples were also tested using a hemagglutination (HA) assay. In brief, 50 µl of positive control (Influenza A/Swine/Iowa/73 (H1N1), negative control (1X PBS, pH = 7.2), and fluid from freeze/thawed CPE-positive wells was added to an untreated 96-well round bottom microtitration plate (Evergreen Scientific, Los Angeles, CA, USA) and serially 2-fold diluted (1:2 to 1:256) in 1X PBS. Thereafter, 50 µl of 0.55% turkey red blood cells (USDA: NVSL) was added to each well and the plates incubated for 30 min at 25°C. A positive reaction produced red blood cell agglutination, i.e., a “mat” at the bottom of the well.

Samples demonstrating CPE on the microinfectivity assay and an HA virus titer ≥1:4 were considered positive for infectious influenza virus. Virus titers were calculated on the basis of the number of wells in the microinfectivity assay showing CPE. Each sample was run in duplicate with the titers averaged.

**Porcine reproductive and respiratory syndrome virus microinfectivity assay**

MARC-145 cells propagated in 96-well tissue culture plates (Corning) using DMEM growth medium were incubated at 37°C in a humidified 5% CO₂ incubator for 72 hr. Samples to be assayed were serially 10-fold diluted (10^{-1} to 10^{-5}) in DMEM growth medium without FBS. The growth medium was discarded and 5 wells were inoculated with 100 µl of sample at each dilution. The plates were placed in a 37°C humidified 5% CO₂ incubator for 90 min, the inoculum was discarded, and 100 µl per well of growth medium containing 4% FBS, rather than 10% FBS was added to each well. Plates were incubated at 37°C in a humidified 5% CO₂ incubator for 24 hrs. Following incubation, cells were fixed with cold, 80% acetone/water solution and stained for 30 min at 37°C with SDOW-17F, fluorescein isothiocyanate-conjugated monoclonal antibody specific for PRRSV diluted according to
manufacturer's instructions (Rural Technologies Inc., Brookings, SD, USA). Virus titers were calculated on the basis of the number of wells showing a PRRSV-specific fluorescence reaction at each dilution. Each sample was run in triplicate and the titers averaged.

**Bovine viral diarrhea virus microinfectivity assay**  
Madin-Darby Bovine Kidney cells (MDBK), (ATCC) were propagated in 96-well plates (Costar) using complete Ex-Cell™ MDBK-MM Medium (Sigma Chemical Co.) containing 0.25 μg ml⁻¹ Amphotericin B (Sigma Chemical Co.), 50 μg ml⁻¹ gentomycin (Sigma Chemical Co.), 2mmol L⁻¹ L-glutamine (Sigma Chemical Co.), 100 IU ml⁻¹ penicillin (Sigma Chemical Co.), 100 μg ml⁻¹ streptomycin (Sigma Chemical Co.) and supplemented with 5% heat-inactivated equine serum (HyClone). Plates were incubated at 37°C in a humidified 5% CO₂ incubator until the cell monolayer was confluent. Samples were serially 10-fold diluted (10⁻¹ to 10⁻⁶) in complete Ex-Cell™ MDBK-MM Medium and 100 μl per well of each sample dilution was applied over the cell suspension. Plates then incubated at 37°C in a humidified 5% CO₂ incubator for 24 hr, then the growth medium was replaced with 200 μl per well of complete medium. The plates were returned to the incubator for an additional 24 hr and viewed for CPE. The plates were then frozen at -80°C until confirmatory testing to be carried out. Confirmatory testing and genotyping of BVDV results was done using RT-PCR with visualization on a 2% agarose-gel (Ameresco, Solon, OH, USA). The viral RNA was extracted as per the manufacturer’s specifications using the Ambion MagMAX™-96 Viral RNA Isolation Kit (Ambion, Austin, TX, USA); reverse transcribed and amplified using the Qiagen® One-Step RT-PCR Kit (Qiagen, Valencia, CA, USA). Thermal cycling conditions were as follows; 50°C for 30 minutes, melt at 95°C for 15 minutes, 35 cycles of amplification at 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute, and extension for 10 minutes at 72°C. Primers used were 5’-TCGACGCCCTTTGAGGT-3’ and 5’-TCGACGCTTTTGAGGACAAGC-3’. The 180 basepair product was run on a 2% agarose gel and visualized under UV illumination.

**Reovirus plaque assay**  
L929 cells in Joklik’s minimum essential medium (Sigma Chemical Co.) containing 10% fetal bovine serum (Atlanta Biological, Lawrenceville, GA,
USA) were added to 6-well plates (Corning) at a concentration of $2 \times 10^6$ cells per well and incubated at $37^\circ C$ overnight. Thereafter, the medium was removed and the cells were washed once with sterile 1X PBS. To conduct the assay, the sample was serially diluted ten-fold, 100 µl per well was added and allowed to adsorb for one hr at $25^\circ C$. The wells were then overlaid with 2 ml of complete 1X Medium 199 at $37^\circ C$. Overlay was formulated as 2X Medium 199 (Sigma Chemical Co.) containing 4 mmol L-1 ml-1 L-Glutamine (Mediatech), 200 IU ml$^{-1}$ penicillin, 200 µg ml$^{-1}$ streptomycin (Mediatech), and 20 µg ml$^{-1}$ trypsin (Mediatech) to which was added to an equal volume of 58°C 2% agar (BD Bacto™, Franklin Lakes, NJ, USA). The plates were incubated for 48 h in a $37^\circ C$ humidified 5% CO$_2$ incubator prior to counting plaques.

**Exposure of viruses to ultraviolet (UV$_{254}$)**

**Source of ultraviolet** The UV$_{254}$ emitting apparatus consisted of an 18 inch (46 cm), 2-lamp, surface-mounted, 110 volt, Ultraviolet fixture (American UV Company, Lebanon, IN, USA) fitted with two standard output, quasi-collimated beam, low pressure, mercury vapor germicidal lamps emitting monochromatic ultraviolet radiation at 253.7 nm (American UV Company). For uniform output, the lamps were "burned in" for 8 hr prior to use. The UV$_{254}$ source was mounted within an environmental chamber capable of maintaining a constant temperature of 4°C (Percival Scientific, Perry, IA, USA). The inside of the environmental chamber was draped with black burlap to eliminate reflection of the UV$_{254}$. To allow lamps to reach full operating pressure within the 4°C environmental chamber, ultraviolet emitting lamps were turned on 2 hr prior to commencing the irradiation experiment and 20 minutes before each exposure treatment, thereafter.

**Virus exposure to ultraviolet** Initial concentrations of $1 \times 10^{8.4}$ (BVDV), $1 \times 10^{6.0}$ (PRRSV), $1 \times 10^{6.0}$ (influenza virus) and $1 \times 10^{9.5}$ (reovirus) TCID$_{50}$ ml$^{-1}$ were exposed to specific UV$_{254}$ doses (25, 50, 75, 100, 125, 150, 200, 250, and 300 mJ/cm$^2$) by placing aliquots (2mm depth) of each virus into 4 separate wells. One positive control virus (either influenza virus Type A, PRRSV, BVDV, or reovirus) was placed in a covered fifth well. The UV$_{254}$ exposure dose (intensity x time) was determined using the integration function of two
commercial radiometers equipped with UV$_{254}$ sensors (Technika, Scottsdale, AZ, USA). The UV$_{254}$ doses (n = 9) were replicated 5 times, which corresponded to 45 plates.

To achieve the pre-determined sample exposure dose, the two center wells in one row of each plate were removed to accommodate a UV$_{254}$ sensor. The viruses were exposed to UV$_{254}$ by the use of an electronically controlled shutter system. After lamp warm-up (20 min), the shutters were opened and the plate exposed to the target dose. When the dose was reached, the UV$_{254}$ emitter was shut off, the shutter system reset, the next plate placed in the environmental chamber, and the UV$_{254}$ emitters warmed up for 20 minutes prior to exposure. Following UV$_{254}$ exposure, each virus suspension was harvested using a pipette, placed in a 2ml storage vial labeled with the virus species and a random number, and frozen at -80°C until titrated for infectious virus.

In accordance with the Lamber-Beers Law, the absorbance of any UV$_{254}$ by the liquid medium, UV$_{254}$ was measured at the top of the 2mm depth of the sample and beneath a quartz cuvett containing an equal (2mm) depth of medium. The measured intensity was corrected for absorbance by the viral medium using Equation 2 (Thurston-Enriquez et al. 2003):

$$I_{\text{average}} = \frac{I_0(1-e^{-a_eL})}{a_eL}$$

Where:

- $I_{\text{average}}$ = average UV$_{254}$ intensity (milliwatts square centimeter$^{-1}$)
- $a_e$ = absorbance of the virus suspension to the base e
- $I_0$ = UV$_{254}$ intensity after passing through solution
- $L$ = depth (centimeters) of the solution irradiated by the UV$_{254}$ energy.
ANALYSIS

Two statistical models were used for analysis the $\text{UV}_{254}$ inactivation of each virus.

Model 1. The one-stage inactivation model (Equation 1) assumed uniform susceptibility of the virus population and utilized simple linear regression (Equation 3), with the log-transformed virus concentration as the response and the dose of $\text{UV}_{254}$ as the explanatory variable:

$$\log_{10} N_t = \log_{10} N_0 - k \cdot \text{Dose}_t$$

Where:
- $N_t$ = quantity of virus in the test sample after treatment with $\text{Dose}_t$
- $N_0$ = quantity of virus in the unexposed control sample,
- $k$ = the inactivation rate
- $\text{Dose}_t$ = average $\text{UV}_{254}$ intensity $\times$ time

In the one-stage inactivation model, the inactivation constant ($k$) is the quotient of the survival fraction ($\frac{N_t}{N_0}$) plotted against the dose. This linear regression model encompasses all of the data points and forces the points into one curve using the classic regression formula, 
$$(-b = \frac{mx}{y}).$$

Model 2. The two-stage inactivation model (Equation 4) assumed two heterogeneous subpopulations: one relatively susceptible and the other relatively resistant to $\text{UV}_{254}$ treatment (Nibert and Fields 1992):
\[ (4) \quad \log_{10} N_t = \log_{10} N_0 + \log_{10} [(1 - f) \cdot 10^{-k_1 \cdot \text{Dose}_t} + f \cdot 10^{-k_2 \cdot \text{Dose}_t}] \]

Where:

- \( N_t \) = quantity of virus in the test sample after treatment with \( \text{Dose}_t \)
- \( N_0 \) = quantity of virus in the unexposed control sample,
- \( f \) = the resistant fraction of the total initial virus population with inactivation rate \( k_2 \)
- \((1 - f)\) = the susceptible virus population fraction with inactivation rate \( k_1 \)
- \( k_1 \) = the inactivation rate of the inactivation curve for the "fast decay population"
- \( k_2 \) = the inactivation rate of the inactivation curve for the "resistant population"
- \( \text{Dose}_t \) = average UV\textsubscript{254} intensity \( \times \) time

The two-stage inactivation model provides two inactivation constants based on the quotient of the survival fractions of two separate microbial populations: the UV-susceptible population and the resistant population. The susceptible population \((1 - f)\) has an inactivation constant of \((K_1)\); the resistant population \( (f) \) has an inactivation constant of \((K_2)\).

The analysis of the data was conducted using SAS\textsuperscript{®} Version 9.2 (SAS\textsuperscript{®} Institute Inc., Cary, NC). Simple linear regression analysis was performed for Model 1 using SAS\textsuperscript{®} procedure GLM and non-linear regression analysis was performed for Model 2 using SAS\textsuperscript{®} procedure NLIN. Lack-of-fit F tests were performed to assess whether the two models fit the data adequately well, by comparing to a full ANOVA model. A \( P \)-value \( \leq 0.05 \) was considered significant.

**RESULTS**

Analysis of the data showed that the one-stage inactivation model did not fit the data (Table 1). Specifically, the lack of fit tests for influenza virus \((P = 0.0009)\), PRRSV \((P = 0.0002)\), BVDV \((P < 0.0001)\), and reovirus \((P = 0.0410)\) showed a statistically significant difference between the observed variation in the data and the variation explained by the single curve model for each virus. Because of the lack of fit, the inactivation constants \((k)\) derived from
the one-stage inactivation analysis cannot accurately describe the amount of UV_{254} needed for inactivation of these viruses under the conditions of the study.

Further analysis of the data showed a good fit between the observed variation in data and the variation explained by the two-stage inactivation model with lack of fit test P-values > 0.05 for all viruses (Table 1). The two-stage model described inactivation curves composed of two slopes separated by an inflection point denoting the inactivation kinetics of the two viral populations (Figures 1 - 4). The two-stage inactivation analysis estimated the susceptible population (1-f) as 98.8% (influenza virus), 94.1% (PRRSV), 99.9% (BVDV), and 87.3% (reovirus) of the total viral population. Inactivation rates (k_1) in the susceptible population were calculated as 0.0651 (influenza virus), 0.4995 (PRRSV), 0.0663 (BVDV), and 0.0142 (reovirus). The resistant population (f) was a low proportion of the total population of influenza virus (1.2%), PRRSV (5.9%), and BVDV (0.07%), but a larger proportion of the reovirus population (12.7%). Compared to the UV_{254}-susceptible population, the resistant populations showed lower inactivation constants (k_2), i.e., 0.0055 (influenza virus), 0.0143 (PRRSV), 0.0095 (BVDV), and 0.0035 (reovirus).

**DISCUSSION**

Chick's law (Harriet Chick, 1908) describes the rate of microbial inactivation as the ratio of the viable population (N_t) to the original microbial population (N_0) in relation to exposure time (t) to an inactivator (Rubin and Elmaraghy 1977). The resulting one-stage inactivation constant (slope) is an estimate of the rate of microbial inactivation based on the assumption of a linear relationship between microbial inactivation and contact time. This relationship has been used to describe the kinetics of microbial populations under a variety of inactivating conditions, including exposure to UV_{254} irradiation (Chang *et al.* 1985; Kowalski *et al.* 2001; Thurston-Enriquez *et al.* 2003). One-stage inactivation estimates are accurate for describing UV_{254} inactivation in homogenous microbial populations (Hiatt 1964). However, one-stage estimates do not address the problem of "tailing off" of UV inactivation curves and may over-estimate the rate of inactivation (Hiatt 1964; Hijnen *et al.* 2006). Jagger (1967) hypothesized that microbial populations were actually composed of two sub-populations; a
larger UV$_{254}$-susceptible population and a smaller UV$_{254}$-resistant population. Shortly thereafter, Riley and Kaufman (1972) proposed an equation that described the UV$_{254}$-inactivation of *Serratia marcescens* as a two-stage process.

Perhaps because deriving estimates for the two-stage inactivation model were computationally demanding prior to availability of desktop computers, the two-stage approach has not been widely used to describe the kinetics of microbial inactivation. For example, there are no estimates of UV$_{254}$ inactivation of BVDV, PRRSV, reovirus strain T3D$^c$ and influenza virus using the two-stage analysis with which to make comparisons to the results reported herein. Importantly, a comparison of one-stage and two-stage inactivation models using experimental data showed that inactivation of the viruses in the study was a two-stage process, as confirmed by a statistical lack-of-fit test. Overall, this study supports the validity of the two-stage approach as a method for evaluating UV$_{254}$ inactivation kinetics. In addition, these results provide guidelines to persons responsible for the prevention and control of these agents in the environment.

**ACKNOWLEDGMENTS**

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### TABLES AND FIGURES

**Table 1.** Parameter estimates and lack of fit test results for two inactivation models

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<th><strong>Influenza virus</strong> *</th>
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<td>-0.4995</td>
<td>-0.0663</td>
<td>-0.0142</td>
</tr>
<tr>
<td>Resistant population proportion ((f))</td>
<td>0.012</td>
<td>0.059</td>
<td>0.001</td>
<td>0.127</td>
</tr>
<tr>
<td>((K_2)) Inactivation rate ((f))</td>
<td>-0.00552</td>
<td>-0.0143</td>
<td>-0.00948</td>
<td>-0.00347</td>
</tr>
<tr>
<td>Lack of fit test (P)-value</td>
<td>0.7146</td>
<td>0.0734</td>
<td>0.2227</td>
<td>0.2807</td>
</tr>
</tbody>
</table>

* Influenza A/Swine/Iowa/73 (H1N1) provided by USDA: National Veterinary Service Laboratories, Ames, IA

† PRRSV Type 2 (isolate MN-184) provided by Dr. Scott Dee, University of Minnesota, St. Paul, MN

‡ Bovine viral diarrhea virus type 2 (Singer strain) provided by Dr. J. Ridpath, USDA: National Animal Disease Center, Ames, IA

§ Reovirus strain T3D\(^c\) provided by Dr. Cathy Miller, Iowa State University, Ames, IA

\(^c\) Kowalski et al., 2000
Figure 1. Influenza virus: Linear regression (Lack of fit $p = 0.0009$, $k = -0.0124$) vs. two-stage inactivation (Lack of fit $p = 0.7146$, $k_1 = -0.0651$, $k_2 = -0.0055$)
Figure 2. PRRSV: Linear regression (Lack of fit $p = 0.0002$, $k = -0.0258$) vs. two-stage inactivation (Lack of fit $p = 0.0734$, $k_1 = -0.4995$, $k_2 = -0.0143$)
Figure 3. BVDV: Linear regression (Lack of fit $p = <0.0001$, $k =-0.0188$) vs. two-stage inactivation (Lack of fit $p = 0.2227$, $k_1 =-0.0663$, $k_2 =-0.0095$)
Figure 4. Reovirus: Linear regression (Lack of fit $p = 0.0410$, $k = -0.0066$) vs. two-stage inactivation (Lack of fit $p = 0.2807$, $k_1 = -0.0142$, $k_2 = -0.0035$)
CHAPTER 5. EFFECT OF TEMPERATURE AND RELATIVE HUMIDITY ON ULTRAVIOLET (UV$_{254}$) INACTIVATION OF AIRBORNE PORCINE RESPIRATORY AND REPRODUCTIVE SYNDROME VIRUS

A manuscript to be submitted to Veterinary Microbiology

Timothy D. Cutler, Chong Wang, Steven J. Hoff, Jeffrey J Zimmerman

ABSTRACT

The objective of this research was to estimate the effects of temperature and relative humidity on the inactivation of airborne porcine reproductive and respiratory syndrome (PRRS) virus by ultraviolet light (UV$_{254}$). Aerosols of PRRS virus were exposed to one of four doses of UV$_{254}$ under nine combinations of temperature (n = 3) and relative humidity (n = 3). Inactivation constants ($k$), defined as the absolute value of the slope of the linear relationship between the survival fraction of the microbial population and the UV$_{254}$ exposure dose, were estimated using the random coefficient model. The associated UV$_{254}$ half-life dose for each combination of environmental factors was determined as $(\log_{10}2/k)$ and expressed as UV$_{254}$ mJ per unit volume. The effects of UV$_{254}$ dose, temperature, and relative humidity were all statistically significant, as were the interactions between UV$_{254}$ dose*temperature and UV$_{254}$ dose*relative humidity. PRRS virus was more susceptible to ultraviolet as temperature decreased; most susceptible to ultraviolet inactivation at relative humidity between 25 and 79%, less susceptible at relative humidity $\leq$ 24%, and least susceptible at $\geq$ 80% relative humidity. The current study allows for calculating the dose of UV$_{254}$ required to inactivate airborne PRRS virus under various laboratory and field conditions using the inactivation constants and UV$_{254}$ half-life doses reported therein.
1.0 INTRODUCTION

Initially described in the late 1980s, porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure in sows, poor growth performance in growing pigs, and respiratory disease in pigs of all ages (Zimmerman et al., 2006). Since its emergence as a clinical entity in the late 1980’s, PRRS virus has proven to be a persistent threat to the health and productivity of pig herds and the economic well-being of pig producers. Neumann et al. (2005) estimated the annual cost of PRRS to U.S. pig producers at $560.32 million per year. By comparison, prior to eradication, annual losses in the U.S. to classical swine fever (hog cholera) and pseudorabies virus were estimated at $364.09 million (Wise, 1981) and $36.27 million (Hallam et al., 1987), respectively (adjusted to Year 2004 dollars).

Since the beginning of the PRRS virus pandemic in the 1980’s, movement of the virus between neighboring herds in the apparent absence of direct contact ("area spread") has been reported (Robertson, 1991). Several epidemiological investigations showed that proximity to infected herds increased the risk of a herd acquiring PRRS virus. Le Potier et al. (1997) found that 45% of herds suspected to have become infected through area spread were located within 500 meters (0.3 miles) of the postulated source herd and only 2% were one kilometer from the initial outbreak. In Denmark it was observed that the likelihood of herd positivity increased as the density and proximity of PRRS virus-positive neighboring herds increased (Mortensen et al., 2002).

In the last decade, researchers have confirmed the occurrence of airborne transmission of PRRS virus over significant distances. Initially, Dee et al. (2005b) demonstrated that infectious airborne PRRS virus could travel over distances ≥150 meters. Thereafter, using a source population of 300 PRRS virus-infected pigs, Dee et al. (2009) demonstrated long-distance airborne movement by the successful recovery of infectious virus up to 4.7 km from the source. Subsequently, Otake et al. (2010) recovered infectious airborne PRRS virus at distance of 9.1 km from the source. Cumulatively, the epidemiological and experimental data suggest that airborne PRRS virus is a significant, and perhaps the primary, means of area spread.
Protection of pig barns from airborne spread of infectious agents is a recent concept. To date, researchers have primarily focused on preventing the introduction of airborne PRRS virus into barns using commercially-available air filters, e.g., high-efficiency particulate air (HEPA) filters, minimum efficiency rating value (MERV) filters of various efficiencies, and fiberglass pre-filters. Overall, the results demonstrated that filtering incoming air with HEPA filters and MERV filters \( \geq 14 \) prevented the transmission of PRRS virus (Dee et al., 2005, 2006a, 2006b, 2010).

Ultraviolet inactivation of PRRS virus may also offer promise. Wheeler et al. (1945) reported the use of ultraviolet to inactivate airborne rubella virus and *Streptococcus pyogenes* in Army and Navy barracks. Likewise, Perkins et al. (1947) reduced the spread of airborne viral pathogens ("measles") in school classrooms using ultraviolet. Riley (1961) demonstrated that ventilated air from hospital tuberculosis wards produced tuberculosis in guinea pigs, but not when the air was irradiated with ultraviolet light. In recent years, UV\(_{254}\) emitters have been engineered into areas where people congregate either by placing UV\(_{254}\) light tube grids into existing ventilation ductwork or by installing free standing UVC emitters (Brickner et al., 2003; Dumyahn and First, 1999; McDevitt et al., 2007; Menzies et al., 1999; Noakes et al, 2006). Ultraviolet technology is appealing due to its low cost as compared to HEPA filtration (Brickner et al., 2003). However, effective implementation must be based on achieving a dose of UV\(_{254}\) sufficient to inactivate the target. No estimates of the effect of UV\(_{254}\) on airborne PRRS virus have been published. Therefore, the objective of this experiment was to evaluate the capability of ultraviolet (UV\(_{254}\)) to inactivate airborne PRRS virus under varying conditions of temperature and relative humidity.

### 2.0 MATERIALS AND METHODS

#### 2.1 Experimental design

The objective of this study was to evaluate the effect of temperature and relative humidity on the inactivation of airborne PRRS virus by ultraviolet irradiation (UV\(_{254}\)). Aerosols of PRRS virus were exposed to four levels of UV\(_{254}\) under nine defined conditions of temperature and relative humidity (Table 1). Each combination of temperature and relative humidity was
replicated 3 times. Samples of air collected after UV\textsubscript{254} treatment were titrated for infectious PRRS virus and the data used to calculate the UV\textsubscript{254} inactivation constants (k) and UV\textsubscript{254} half-life (T\textsubscript{1/2}) exposure doses for each combination of temperature and relative humidity.

Table 1. Temperature and relative humidity combinations of PRRS virus aerosols

<table>
<thead>
<tr>
<th>Relative Humidity</th>
<th>Airborne PRRS virus received 4 levels of UV\textsubscript{254} treatment at each combination of temperature and relative humidity. Each combination was replicated 3 times.</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 15°C</td>
<td><strong>Temperature</strong> &lt; 15°C 16°C to 29°C ≥ 30°C</td>
</tr>
</tbody>
</table>

2.2 Porcine reproductive and respiratory syndrome virus
A type 2 PRRS virus isolate, MN-184 (kindly provided by Dr. Scott Dee, University of Minnesota, MN, USA) was propagated on MARC-145 cells, a clone of the African monkey kidney cell line MA-104 (Kim et al., 1993). Cells were grown in 162 cm\textsuperscript{2} flasks (Corning Incorporated, Corning, NY, USA) using growth media; Dulbecco’s Modified Eagles Medium (DMEM), (Mediatech Inc., Manassas, VA, USA) supplemented with 0.25 μg/ml Amphotericin B (Sigma Chemical Co., St. Louis, MO, USA), 50 μg/ml gentamicin (Sigma), 0.5 M L-glutamine (Fisher Scientific, Hampton, NH, USA), 300 international units (IU) per ml penicillin (Sigma), 300 μg/ml streptomycin (Sigma), 1.0% nonessential amino acids (HyClone, Logan, UT, USA), 25 mM HEPES buffer (Sigma Chemical Co.) and 10.0% heat-inactivated fetal bovine serum (FBS) (Sigma Chemical Co.). When cells were confluent (72 to 84 hrs), the medium was discarded and the flasks inoculated with 5 ml DMEM (without L-glutamine) containing PRRS virus isolate MN-184 at a virus titer of 1 x 10\textsuperscript{3.5} median tissue culture infective dose (TCID\textsubscript{50}) per ml. Flasks were placed on a rocking platform in a 37°C humidified 5% CO\textsubscript{2} incubator for 90 min and then 40 ml of supplemented DMEM (Mediatech Inc.) growth medium (now with 4% FBS) was added and the flasks returned to the incubator for 36 hrs. Cell culture supernatant was harvested by flask freeze-thaw and centrifugation (3000 x g for 20 minutes at 4°C). Virus stock was stored in 25 ml aliquots at -
2.3 Experimental procedures

2.3.1 Overview The system was constructed such that, throughout the 45 min experiment, aerosolized PRRS virus continuously flowed from Reservoir One to Reservoir Two and then across a UV$_{254}$ exposure field. A manifold in Reservoir Two functioned to equally distribute aerosolized PRRS virus into four quartz tubes placed parallel to each other in the field of ultraviolet irradiation. Each quartz tube represented a different level of UV$_{254}$ treatment.

Airflow through the system was impelled by negative pressure generated by four AGI-30 glass impingers (Ace Glass, Vineland, NJ, USA), each operating at a flow rate of 12.5 L per min. Thus, air flow through the system totaled 50 L per min. Temperatures in Reservoir One and Reservoir Two were adjusted to achieve targeted temperatures and relative humidities at the UV$_{254}$ irradiation field. Air samples collected by the impingers downstream of the UV$_{254}$ exposure field were titrated for infectious PRRS virus.

2.3.2 Aerosolized PRRS virus The virus solution to be aerosolized consisted of 25 ml of stock PRRS virus ($1 \times 10^7$ TCID$_{50}$ per ml), 50 ml of sterile 1X PBS (Thermo Fisher, Rockford IL, USA), and 0.1% (v/v) antifoam A emulsion (Sigma Chemical Co., A5758). Previous research showed that antifoam A emulsion innocuous for cultured cells and PRRS virus (Hermann et al., 2006). The virus solution was maintained on ice and shielded from light until nebulization. The solution was aerosolized using a 24-jet Collison nebulizer (BGI Inc., CN60, Waltham, MA, USA) operating on compressed air at 1.55 kg per cm (22 pounds per square inch). Under these parameters approximately 1.0 ml of virus solution was nebulized each minute into particles of approximately 1.9 µm diameter (Hermann et al., 2007).

2.3.3 Relative humidity and temperature For each 45 min replicate, virus was continuously nebulized into Reservoir One (16 L; aerosol residence time ~19 sec). Reservoir One was housed in a chamber capable of maintaining temperatures between -20°C and room
temperature (Carroll Coolers, Inc., Carroll, IA). Air was drawn from Reservoir One into Reservoir Two (13 L; aerosol residence time ~16 sec). Reservoir Two was housed in a chamber capable of maintaining temperatures between 0°C and 40°C (SS Series 600, 1695-03-36231, Terra Universal, Inc., Anaheim, CA, USA).

Based on the rate of nebulization (1.0 ml per minute at 1.55 kg per cm) and the temperature capabilities of Reservoirs One and Two, psychrometric calculations determined that the system was theoretically capable of producing an air flow with the RH (%) values listed in Table 2, with temperature at the UV$_{254}$ field of exposure approximating the temperature in Reservoir Two.

For each replicate, the actual temperature and relative humidity of the aerosol was measured (Vaisala, HMI41 indicator and HMP46 temperature probe, Helsinki, Finland) inside a manifold located within Reservoir Two and immediately upstream of the UV$_{254}$ exposure field (Table 1). Temperature and relative humidity measurements were taken prior to starting the replicate and then at the beginning, midpoint and end of each replicate to confirm that the system operated at targeted parameters. All targeted combinations of temperature (n = 3) and relative humidity (n = 3) were conducted in triplicate for a total of 9 replicates (Table 1).

<table>
<thead>
<tr>
<th>Reservoir One (°C)</th>
<th>-20</th>
<th>-15</th>
<th>-10</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
<td>31</td>
<td>47</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>22</td>
<td>32</td>
<td>70</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>10</td>
<td>16</td>
<td>23</td>
<td>50</td>
<td>71</td>
<td>100</td>
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<td>30</td>
<td>40</td>
<td>55</td>
<td>75</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2. Calculated relative humidity at the UV$_{254}$ field of exposure

<table>
<thead>
<tr>
<th>Reservoir Two (°C)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
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<th>25</th>
<th>30</th>
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<tr>
<td>0</td>
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<td>5</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
2.3.4 Ultraviolet (UV\textsubscript{254}) field of exposure  

The UV\textsubscript{254} emitting apparatus consisted of six low-pressure, mercury-vapor discharge lamps 28.8 cm in length (American UV Company, Lebanon, IN, USA). Lamps were mounted in three 2-lamp, reflective, surface-mounted, 110 volt, fixtures (American UV Company, Lebanon, IN, USA). To avoid fluctuation in UV\textsubscript{254} intensity, lamps were operated at their maximum UV\textsubscript{254} emission capacity for 10 min before the start of each experiment. UV\textsubscript{254} emission was measured using three calibrated radiometers (Model 1700, International Light Inc. Newburyport, MA, USA; VLX3W Technika, Phoenix, AZ, USA).

Exposure of airborne PRRS virus to UV\textsubscript{254} was done by passing the airborne virus through 4 quartz tubes (10mm internal diameter x 12.75 external diameter x 14cm in length) connected to a manifold within Reservoir Two. Quartz tubes were placed 25 cm from, and parallel to, the UV254 emitters. Between each replicate, quartz tubes were cleaned with commercial quartz cleaner following the manufacturer’s recommendations (Hellmanex® II, Hellma GmbH & Co., Mülheim, Germany). Quartz tubing was evaluated for cleanliness and UV\textsubscript{254} absorbance prior to each replicate by measuring UV\textsubscript{254} intensity beneath and beside the tubing with the impingers in operation (mock aerosol). No differences in the two measurement were detected. Therefore, UV\textsubscript{254} exposure dose measured directly below the quartz tubing.

2.3.5 Ultraviolet (UV\textsubscript{254}) treatment of PRRS virus aerosol  

Each of the four quartz tubes delivered a different UV\textsubscript{254} treatment. This was achieved by shielding all but a specific length of each tube, i.e., 1.3 cm (residence time of 0.07 sec); 3.2 cm (residence time of 0.14 sec); and 5.2 cm (residence time of 0.25 sec). A completely shielded tube served as a non-exposed (positive) control. Treatment (shielding) was randomized to quartz tubes before each replicate. UV\textsubscript{254} irradiance was measured at the unshielded area of each quartz tube at the start, midpoint, and end of each replicate and averaged. For each treatment, the UV\textsubscript{254} dose delivered to airborne PRRS virus was calculated as:
\[ D = I \times T \]

Where:
- \( D \) = Ultraviolet dose (mJ per cm\(^2\))
- \( I \) = Irradiance (mW per cm\(^2\)). Average of irradiance measured at start, midpoint, and end of each replicate.
- \( T \) = Residence time (sec)

Based on the parameters described above, the mean \( UV_{254} \) exposure doses across all replicates for the four treatments were calculated as: zero for the non-exposed (positive) control, 0.05 (S.D. 0.009) mJ per cm\(^2\); 0.12 (S.D. 0.016) mJ per cm\(^2\), and 0.20 (0.039) mJ per cm\(^2\).

### 2.3.6 Sampling of \( UV_{254} \)-treated airborne PRRS virus

Each quartz tube was independently connected to an all-glass impinger (AGI-30, Ace Glass Inc., Vineland, NJ, USA) containing 25 ml of 1X PBS and shielded against ultraviolet. Impingers were placed on ice throughout the 45 minute experiment to avoid dessication of PBS and preserve virus viability. Impingers were operated at a constant flow rate of 12.5 L per min. Vacuum pressure was maintained using oil-less pumps (Fisher Scientific, S413801, Hampton, NH). All connections were sealed and checked for leakage prior to each replicate. Air from the system was exhausted through a biosafety level 2 (BL2) cabinet (NuAire Laboratory Equipment Supply, Plymouth, MN, USA).

### 2.3.7 PRRS virus microinfectivity assay (TCID\(_{50}\))

Impinger collection fluid was assayed for the concentration of infectious PRRS virus immediately following each replicate. Virus titrations were done on confluent monolayers of MARC-145 cells in 96-well plates (Corning Incorporated). Cell monolayers were prepared by inoculating 100 \( \mu \)l of cells suspended in growth medium into each well, and then incubating the plates at 37\(^\circ\)C in a humidified 5\% \( CO_2 \) incubator for 24 hrs. Each sample was serially 10-fold diluted in DMEM, with five wells were inoculated with 100 \( \mu \)l of each sample dilution. Thereafter, plates were incubated at 37\(^\circ\)C in a humidified 5\% \( CO_2 \) incubator for 2 hrs, after which the
inoculum was discarded and 100μl per well of DMEM supplemented with 4% FBS was added. Plates were incubated at 37°C in a humidified 5% CO2 incubator for 24 hrs, after which the cells were fixed with aqueous 80% acetone solution and stained with a fluorescein isothiocyanate-conjugated monoclonal antibody specific for PRRS virus (SDOW17, Rural Technologies, Inc., Brookings, SD, USA). Virus titers were calculated using the Spearman-Kärber method on the basis of the number of wells showing PRRS virus-specific fluorescence at each dilution and expressed as tissue culture infection dose 50 (TCID50) per ml of impinger fluid.

3.0 ANALYSIS

To analyze the main effects (UV254 dose, temperature, and relative humidity) and their interactions, the TCID50 data were log10-transformed and analyzed using a random-coefficient ANCOVA (analysis of covariance) model with the quantitative explanatory variable "UV254 dose" and categorical explanatory variables of "temperature" and "relative humidity" in SAS® Version 9.2 (SAS® Institute Inc., Cary, NC, USA). Inactivation constants (k), defined as the absolute value of the slope describing the linear relationship between the survival fraction of the microbial population and the UV254 exposure dose (Goldberg et al., 1958), were estimated using the random coefficient model. The associated UV254 half-life (T1/2) dose for each combination of environmental factors was determined as (log102/k) and expressed as UV254 mJ per unit volume.

4.0 RESULTS

The effects of UV254 dose, temperature, and relative humidity on PRRS virus (TCID50) recovered in air sample were all statistically significant (p < 0.001), as were the interactions between UV254 dose*temperature (p = 0.0475), and UV254 dose*relative humidity (p = 0.0204). Inactivation constant estimates and UV254 half-life dose estimates are given in Table 3. Comparisons among the three temperature groups detected a significant difference in the UV254 inactivation constant for PRRS virus at low vs. high temperatures (p = 0.0167), but not between low vs. medium (p = 0.1044) or medium vs. high temperatures (p = 0.4635).
Comparisons among the three levels of relative humidity revealed a statistically significant difference in inactivation constants between medium vs. high relative humidity ($p = 0.0060$), but not between and low vs. medium ($p = 0.0700$) or low vs. high relative humidity ($p = 0.3291$).

**Table 3. Inactivation constants and UV$_{254}$ half-life doses by temperature and relative humidity**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Relative Humidity</th>
<th>≤ 24%</th>
<th>25% to 79%</th>
<th>≥ 80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 15°C</td>
<td>Main effects</td>
<td>4.25 (0.071)*</td>
<td>5.87 (0.051)</td>
<td>3.41 (0.088)</td>
</tr>
<tr>
<td></td>
<td>5.68 (0.053)</td>
<td>5.42 (0.055)</td>
<td>7.04 (0.043)</td>
<td>4.58 (0.070)</td>
</tr>
<tr>
<td>16°C to 29°C</td>
<td>4.25 (0.071)</td>
<td>3.99 (0.075)</td>
<td>5.61 (0.054)</td>
<td>3.15 (0.096)</td>
</tr>
<tr>
<td>≥ 30°C</td>
<td>3.59 (0.084)</td>
<td>3.33 (0.090)</td>
<td>4.96 (0.061)</td>
<td>2.49 (0.121)</td>
</tr>
</tbody>
</table>

*Inactivation constant and (half-life). Inactivation constant (k) is the absolute value of the slope of the PRRS virus survival fraction and the UV$_{254}$ exposure dose. Larger k values indicate more rapid PRRS virus inactivation. Half-life dose is expressed as uv$_{254}$ mj per unit volume.

5.0 DISCUSSION

In this study, UV$_{254}$ inactivation constants for PRRS virus were derived for three temperature ranges, three relative humidity ranges, and their combinations. The effects of temperature and relative humidity on the UV$_{254}$ inactivation of PRRS virus were statistically significant, but the interaction of temperature and relative humidity was not. Ultraviolet inactivation constants decreased in a linear fashion as temperature increased, with statistically significant differences in inactivation constants detected in low vs. high temperatures, but not between low vs. medium or medium vs. high temperatures. The effect of relative humidity on UV$_{254}$
inactivation was more complex. For any temperature, the rate of PRRS virus inactivation was highest at relative humidity between 25% and 79% and lowest at relative humidity ≥ 80%. These observations were reflected in statistically significant differences in inactivation constants in medium vs. high relative humidity, but not between other comparisons.

A search of the refereed literature found no publications describing UV\textsubscript{254} inactivation of airborne viruses under varying conditions of relative humidity and temperature and only two publications on UV\textsubscript{254} inactivation of airborne viruses under varying conditions of relative humidity. In agreement with the results of this study, Tseng and Li (2005) reported that UV\textsubscript{254} inactivation of four bacteriophages (MS2, phi X174, phi 6, T7) decreased as relative humidity increased at temperatures of 25°C to 28°C and speculated that decreased UV\textsubscript{254} susceptibility under higher relative humidity conditions resulted from attenuation of UV\textsubscript{254} by water sorption onto the viral surface. In contrast, in a study involving bacteriophage MS2, respiratory adenovirus serotype 2, and mouse hepatitis virus (coronavirus), Walker and Ko (2007) reported that UV\textsubscript{254} inactivation increased as relative humidity increased (temperature conditions not reported). Walker and Ko (2007) hypothesized that increased UV\textsubscript{254} susceptibility at higher relative humidity could have been a function of larger droplet size at higher relative humidities. Given the overall paucity of data, fruitful hypothesis generation regarding the mechanisms underlying the interactions between UV254 inactivation, temperature, and relative humidity must await additional data on the ultraviolet inactivation of a greater diversity of micro-organisms.

The current study allows for calculating the dose of UV\textsubscript{254} required to inactivate airborne PRRS virus under various laboratory and field conditions using the inactivation constants and their associated UV\textsubscript{254} half-life doses from Table 3. Since activation constants vary by temperature and relative humidity, a conservative estimate of the necessary UV\textsubscript{254} dose can be made using the smallest inactivation constant (k = 2.49, Table 3) and its associated UV\textsubscript{254} half-life dose. The percent of the infectious viral population remaining after \textit{n} UV\textsubscript{254} half-life doses may be described as \((1/2^n)\); thus, the percent of infectious PRRS virus remaining after 10 UV\textsubscript{254} doses may be calculated as \((1/2^{10}) = 0.1\%\). From this, the dose of UV254 required
to inactivate 99.9% of airborne PRRS virus may be calculated using figure from Table 3 as 
(0.121 mJ per unit volume/half-life) \times (10 \text{ half-lifes}) = 1.21 \text{ mJ per unit volume}. This 
exposure dose may be achieved under different conditions of ultraviolet intensity and 
airflow. As given by the Bunsen-Roscoe Law of Reciprocity, if achieved, this dose will be 
effective regardless of UV_{254} intensity or residence time (Riley and Kaufman, 1972).

ACKNOWLEDGMENTS

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the PRRS CAP, USDA NIFA Award 2008-55620-19132.

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application of ultraviolet germicidal irradiation to control transmission of airborne 

preventing aerosol transmission of porcine reproductive and respiratory syndrome 

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CHAPTER 6. GENERAL CONCLUSIONS

Ultraviolet (UV$_{254}$) inactivation has a long history of use for the disinfection of water and, since the publication of Wells and Wells in 1938, for the disinfection of airborne pathogens of humans and animals. Overall, the research described in this dissertation has supported the historical assessment, i.e., has shown that PRRS virus is susceptible to UV$_{254}$ inactivation at the benchtop. However, to implement UV$_{254}$ on a large scale level will take extensive research. In an agricultural application, the small air volumes and the exposure areas used in the experimental setting must be massively scaled up. To consistently and safely deliver the required UV$_{254}$ dose on the farm will present problems not previously addressed. Other significant challenges stand between ultraviolet technology and its routine application for the disinfection of air would include the following:

1. The first challenge is the nomenclature associated with ultraviolet inactivation. That is, there are a variety of interpretations of the terms used in the design and implementation of ultraviolet light inactivation; indeed each field has its own terminology and definitions. For example, the word “ultraviolet dose” can mean the amount of energy that a target is exposed to (photobiology); the quantum yield, i.e. the number of photons absorbed (photochemistry); or fluence measured in watts/cm$^2$ (engineering) (Rahn et al., 2005; Riley, 1994; Thurston-Enriquez, et al., 2003). These differences in terminology complicate and obfuscate communications across disciplines. Therefore, more rapid development and routine implementation of UV$_{254}$ necessitates the development of uniform terminology.

2. The second challenge is the scarcity of estimates on the level of inactivation required to prevent transmission. This question can only be answered by determining the aerosol median infectious dose (ID$_{50}$) for each airborne pathogen. In fact, the ID$_{50}$s for most airborne pathogens is not known and from the research described in this dissertation we know that isolates of the same viral species may have vastly divergent ID$_{50}$s. Indeed, VanOsdell and Foarde, (2002), state that this missing information is the main factor in preventing the design and implementing UV$_{254}$ disinfection systems on a large scale.
3. From the engineering perspective, the environmental conditions of air temperature, relative humidity, air flow rate, and proper emitter type are critical to the efficacy of UV$_{254}$ for the control of microorganisms (VanOsdell and Foarde, 2002). To achieve targeted levels of microbial inactivation in a flow through system, emitter type must be paired with environmental conditions (VanOsdell and Foarde, 2002). Furthermore, UV$_{254}$ inactivation is affected by airborne particulate matter that shields microorganisms from UV$_{254}$. This is a concern as the particulate matter not only shields the microbes but also coats the UV$_{254}$ emitters. Therefore, an air filtration system should be employed to remove the particulate matter prior to UV$_{254}$ exposure. This, by itself forces UV inactivation to be a system that is used in tandem with other applications and not as a stand-alone application.

Perhaps in part, the use of ultraviolet for the inactivation of microorganisms is appealing because it is a familiar, commercially-available technology and avoids the use of chemicals. Recent interest in this approach for the control of bioaerosols has also been stimulated by concerns for agro- or bio-terrorism. However, based on the complexity of this field of research and given the current development of the technology, the routine use of ultraviolet for the inactivation of microorganisms in aerosols awaits further development. Because of the limitations of the technology described above, ultraviolet may ultimately only function effectively in tandem with other technologies, e.g., downstream of filtration systems or in photocatalytic systems.
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