

**Quantitative studies on airborne transmission of porcine reproductive and
respiratory syndrome virus**

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Dedication

To my wife, Danielle, thank you for your patience, love, and support throughout this program
of study.

To my children, Lily and Claire, your smiles and laughter touch my heart and brighten each
day.

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped RNA virus in the family *Arteriviridae* within the order *Nidovirales* (Cavanagh, 1997). PRRSV transmission of virus within and between swine herds results in reoccurring episodes of reproductive and/or respiratory disease (Baysinger et al., 1997). Transmission of PRRSV to susceptible animals may occur through direct contact between pigs or indirectly via an intermediary route. Direct transmission includes intranasal, intramuscular, oral, intrauterine, and vaginal routes of exposure (Benfield et al., 2000b; Christianson et al., 1993; Gradil et al., 1996; Magar et al., 1995; Magar and Larochelle, 2004; van der Linden et al., 2003; Yaeger et al., 1993). Indirect transmission includes spread by fomites (Dee et al. 2002), arthropods (Otake et al. 2002), and aerosols. Questions regarding the role aerosol transmission of PRRSV plays in disease spread remain unanswered. To ascertain answer to these questions, five quantitative studies on the factors influencing the likelihood of transmission: 1) virus excretion, 2) stability of aerosolized virus, and 3) infectious dose required were conducted.

The objective of the first study was to optimize sampling parameters for increased recovery and detection of airborne PRRSV and swine influenza virus (SIV). Collection media containing antifoams, activated carbons, protectants, and ethylene glycol were evaluated for direct effects on factors impacting the detection of PRRSV and SIV, including virus infectivity, viability of continuous cell lines used for the isolation of these viruses, and performance of reverse-transcriptase polymerase chain reaction (RT-PCR) assays. The results showed that specific compounds influenced the likelihood of detecting PRRSV and

SIV in collection medium. A subsequent study evaluated the effects of collection medium, impinger model, and sampling time on the recovery of aerosolized PRRSV using a method for making direct comparisons of up to six treatments simultaneously. The results demonstrated that various components in air sampling systems, including collection medium, impinger model, and sampling time, independently influenced the recovery and detection of PRRSV and/or SIV. Based on the results of these experiments, the air sampling parameters were optimized for sampling i.e. recovery/detection of PRRSV and implemented throughout the remaining experiments.

The objective the second study was to estimate the analytical sensitivity (detection threshold) of each of four air samplers (AGI-30, AGI-4, SKC BioSampler, and Midwest Micro-Tek sampler) for PRRSV and SIV. In a 5 minute sampling period under controlled conditions, the analytical sensitivity of the AGI-30, AGI-4, SKC BioSampler®, and Micro-Tek samplers for PRRSV was calculated at $1 \times 10^{1.1}$, $1 \times 10^{1.3}$, $1 \times 10^{1.1}$, and $1 \times 10^{1.2}$ TCID₅₀ equivalents and $1 \times 10^{1.4}$, $1 \times 10^{1.1}$, $1 \times 10^{1.6}$, for SIV. Despite marked differences in sampler design, no statistically significant difference in analytical sensitivity was detected between samplers for collection of artificially produced aerosols containing cell culture propagated PRRSV or SIV. Detection thresholds obtained from this experiment were used for reference in excretion studies.

The objective of the third study was to characterize the excretion of these pathogens in respirations from acutely infected pigs. Pigs were inoculated under experimental conditions with one pathogen. Samples were collected from the upper respiratory tract and respiratory exhalations. All pathogens were detected in the upper respiratory tracts of inoculated pigs, but only *M. hyopneumoniae* and *B. bronchiseptica* were detected in expired

air from individually-sampled, acutely-infected pigs. These findings suggested either that acutely infected pigs did not aerosolize PRRSV, PCV-2, SIV, or PRCV, or that the quantity of virus excreted was below the analytical sensitivity (detection threshold) of current sampling and/or assay systems at the individual pig level.

The objective of the forth study was to describe the stability of airborne infectious PRRSV as a function of temperature and relative humidity. A cloud of infectious PRRSV was aerosolized using a 24-jet Collison nebulizer into a dynamic aerosol toroid (DAT) maintained at a specific temperature and relative humidity. The PRRSV cloud within the DAT was sampled repeatedly over time using SKC BioSampler® impingers and the total viral RNA (RT-PCR) and concentration of infectious PRRSV (TCID₅₀) in the air samples was determined. As measured by quantitative RT-PCR, PRRSV RNA was stable under the conditions evaluated in this study. Thus, a comparison of viral RNA and Rhodamine B dye, a physical tracer, found no significant difference in the slopes of the lines. Titers of infectious virus were plotted by time and the half-life (T_{1/2}) of infectious PRRSV was calculated using linear regression analysis. An analysis of the results showed that aerosolized PRRSV was more stable at lower temperatures and/or lower relative humidity, but temperature had a greater effect on the T_{1/2} of PRRSV than relative humidity. Based on these results, an equation was derived to predict the T_{1/2} of infectious airborne PRRSV for any combination of environmental temperature and relative humidity.

The objective of the fifth study was to describe and validate a method that provides improved estimates of the probability that exposure to a specific dose of an airborne infectious pathogen will result in infection in animals. To validate the proposed method, 7 replicates of 10 pigs per replicate were conducted. Pigs were exposed to specific doses of

airborne PRRSV. Following exposure, animals are individually housed and monitored for evidence of infection. The relationship describing the probability of infection after exposure was modeled on the proportions of pigs that became infected by exposure to specific doses. A dose response curve was derived using logistical models. Based on this methodology dose-response curves may be derived for a number of animal host and infectious pathogens. This information is useful in estimating the likelihood of infection associated with exposure to airborne infectious microorganisms.

CHAPTER 1. GENERAL INTRODUCTION

Thesis Organization

This thesis consists of seven chapters. Chapter 1 is the general introduction and review of the literature, “Review of aerosol transmission of aerosol transmission of porcine reproductive and respiratory syndrome virus (PRRSV)”. Chapter 2, “Optimization of a sampling system for the recovery and detection of airborne porcine reproductive and respiratory syndrome virus and swine influenza virus” is published in Applied and Environmental Microbiology. Chapter 3, “Analytical sensitivity of air samplers based on uniform point source exposure to airborne porcine reproductive respiratory syndrome and swine influenza viruses” is published in the Canadian Journal of Veterinary Research. Chapter 4, “Detection of respiratory pathogens in air samples from acutely infected pigs” is published in the Canadian Journal of Veterinary Research. Chapter 5, “Effect of temperature and relative humidity on the stability of infectious porcine reproductive and respiratory syndrome virus in aerosols” is published in Veterinary Research. Chapter 6, “A method to provide improved dose-response estimates for airborne pathogens in animals: An example using porcine reproductive and respiratory syndrome virus” is submitted for publication in Veterinary Microbiology. References and tables for each research paper follow the discussion section of each paper. The last chapter contains the general conclusions of the research studies.

Review of Literature

A critical review of the scientific literature regarding aerosol transmission of porcine reproductive and respiratory syndrome virus

Introduction

First described in 1991 (Terpstra et al., 1991; Wensvoort et al., 1991), porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped RNA virus in the family *Arteriviridae* within the order *Nidovirales* (Cavanagh, 1997). In common with other arteriviruses, PRRSV exhibits a narrow host range (Vogel, 1991; Hopper et al. 1994; Zimmerman et al. 1997) and continual genetic modification of the viral genome during replication (Allende et al., 1999; Chang et al., 2002; Kapur et al., 1996; Le Gall et al., 1998; Rowland et al., 1999). In swine, PRRSV replicates in myeloid cells, principally macrophages (Thanawongnuwech et al., 2000; Wensvoort et al., 1991) and produces a persistent infection in which infectious virus may be recovered from lymphoid tissues for several months (Allende et al., 2000; Benfield et al., 2000a; Christopher-Hennings, et al., 1995; Horter et al., 2002; Wills et al., 1997a; Wills et al., 2003; Zimmerman et al., 1992). Transmission of PRRSV occurs by direct contact between pigs or indirectly via intermediary routes. Direct transmission may involve intranasal, intramuscular, oral, intrauterine, or vaginal exposure to the virus (Benfield et al., 2000b; Christianson et al., 1993; Gradil et al., 1996; Magar et al., 1995; Magar and Larochelle, 2004; van der Linden et al., 2003; Yaeger et al., 1993). Indirect transmission includes virus exposure via fomites (Dee et al. 2002; Otake et al. 2002a), arthropods (Otake et al. 2002b), and aerosols. Once introduced into

commercial swine herds, PRRSV typically becomes endemic with cyclic transmission of virus within the population and reoccurring episodes of reproductive and/or respiratory disease (Baysinger et al., 1997; Carvajal, 2003; Dee and Joo, 1994; FitzSimmons and Daniels, 2003; Keffaber et al., 1992; Thanawongnuwech et al., 2003).

Since the beginning of the PRRSV pandemic in the 1980's, the virus' seeming ability to move between neighboring herds without direct contact has frustrated prevention and control measures (Robertson 1991). This pattern of transmission, commonly termed "area spread," is defined by the entry of PRRSV into a herd in the absence of a factor or event that could explain the introduction of the virus, e.g., movement of pigs, semen, fomites, or humans between herds (Larochelle, 2003). Although the route(s) or frequency of area spread occurs is sparsely documented, airborne transmission has long been postulated to play a major role (Keffaber 1989; Anon 1991).

At present, approximately 65% of U.S. herds are infected with PRRSV, with production losses associated with clinical PRRS estimated to cost U.S. swine producers in excess of US\$560 million annually (Neumann et al., 2005). This cost exceeds the losses attributed to either classical swine fever or pseudorabies viruses (Neumann et al., 2005) prior to their elimination from U.S. swine herds in 1978 (Wise, 1981) and 2004 (AVMA, 2005), respectively. For this reason, it has been proposed that elimination of the virus from North American herds is the best solution for controlling PRRS. However, enthusiasm for PRRSV elimination is tempered by the recognition that, once free of the virus, individual herds and/or regions are at risk of re-infection by area spread. For this reason, understanding the role aerosol transmission plays in re-infection of PRRSV-free herds is critical for future efforts to

control and eliminate PRRSV. To that end, the objective of this paper is to critically review the literature on aerosol transmission and its role in the spread of PRRSV.

Early outbreak investigations

Although PRRSV was present in swine herds in Canada by 1979 (Carmen et al., 1995), it was unrecognized prior to reports from the U.S. of catastrophic outbreaks characterized by severe reproductive losses, extensive post-weaning pneumonia, reduction of growth performance, and increased mortality (Hill, 1990; Zimmerman et al., 1997). In Europe, outbreaks similar to those in the U.S. were reported in November 1990 near Münster, Germany (OIE, 1992). In spite of the implementation of control measures, the disease spread rapidly: outbreaks were reported in the Netherlands and Spain in January 1991, Belgium in March 1991, Great Britain in May 1991 (Edwards et al., 1992), and France in November 1991 (Baron et al., 1992; OIE, 1992).

Clinical PRRS was first described in the U.S., but the earliest and most complete field outbreak investigations were conducted in Europe. These early reports identified PRRS-affected herds using case definitions because the etiology of the disease had not yet been determined and the diagnostic tools necessary to detect PRRSV infection and establish herd infection status had not yet been developed. For example, the case definition used by Cromwijk (1991) identified PRRS-affected herds by the presence of two or more of the following clinical signs within a 14 day period: an increase of $> 8\%$ in abortion and/or premature farrowings, an increase of $> 20\%$ in stillbirths, and piglet mortality $> 25\%$ in the first week post farrowing.

Case definitions, while similar, were developed independently by investigators for their area or region of focus. There was not a single, standardized case definition. The fact that case definitions were the only tool available to identify PRRS-affected herds undoubtedly led to misclassification errors both because the clinical signs used in the case definition were not unique for PRRSV and because, as was subsequently documented (Morrison et al., 1992), herds could be infected with PRRSV and not demonstrate overt clinical disease. Perhaps as a consequence, analyses of the data collected during this period produced reports both affirming and refuting airborne transmission of PRRSV (Komijn et al. 1991; Robertson 1991; Varewyck 1991; Vogel et al. 1991). As was succinctly reported in the Veterinary Record at the time (Anon, 1992), “Opinion is divided about the role of airborne infection.”

Field studies

The situation began to clarify with the isolation and identification of PRRSV in 1991 (Terpstra et al., 1991; Wensvoort et al., 1991). The ability to cultivate the virus *in vitro* led to the development of methods to detect PRRSV and anti-PRRSV antibodies, e.g., immunohistochemistry (Benfield et al. 1992), immunoperoxidase monolayer assay (Wensvoort et al., 1991), indirect fluorescent antibody test (Yoon et al., 1992), serum-virus neutralization test (Benfield et al., 1992), and enzyme-linked immunosorbent assays (Albina et al., 1992). It took some time for the diagnostic assays to become widely accessible, but their existence addressed many of the shortcomings of the early outbreak investigations and improved the quality of the data in epidemiological field studies.

France Among the earliest PRRSV field studies was an epidemiological study of swine herds enrolled in the voluntary sanitary program implemented in the 'Pays de la Loire' region of France (Albina et al., 2003; Le Potier et al., 1997). Following the first clinical PRRS outbreak in the area in November 1992, a serological survey was conducted to estimate the prevalence of infection in the region. Of 2,200 total farms, 733 (33%) were randomly sampled for serological testing and 15 were found to be infected (2%). In 1993, a plan was initiated for the purpose of eliminating PRRSV from the region. Extensive serological monitoring was conducted as part of the project to identify infected herds and then link them to the source of infection. In addition to routine monitoring, herds with any epidemiological associations to infected herds and herds located within 2 km of infected farms were tested at 3 month intervals. Control measures were put in place to prevent the transmission of PRRSV via purchased semen, animals, and fomites (transport vehicles, feed, etc). Although herds continued to become infected, concurrent elimination of PRRSV from infected herds maintained the prevalence of the infection below 2% for over 8 years (1993 to 2000). Among the 118 herds that became infected, the investigators attributed the introduction of PRRSV to the introduction of pigs (56%, n = 66), semen (19.5%, n = 23), fomites or slurry (21.2%, n = 25), and unknown (3.4%, n = 4).

As the first sustained regional effort at control and elimination of PRRSV, this project is noteworthy for its success. This was not a research project on transmission and questions on area spread and/or aerosol transmission are left unresolved. For example, the authors state that "The prevalence of infected herds suspected to have been contaminated from nearby herds was 45% under 500 m and only 2% over 1 km from the initial outbreaks", but the number of herds infected by distance is not provided, nor is it clear how contamination from

nearby herds relates to the routes of transmission previously described, i.e., introduction via pigs, semen, fomites, and unknown. The study is also limited by the fact that some microbiological tools had not become readily available, e.g., virus sequencing may have provided further clarification of the question of area spread.

Denmark PRRS was diagnosed in Denmark in 1992 (Mortensen and Madsen, 1992; Bøtner et al., 1994) and within 60 days spread to 27 herds in the southern region. Because of trade restrictions, the virus was assumed to have entered Denmark via airborne transmission of the virus from infected herds in Germany. By 1996 the virus had spread to ~25% of the Danish sow herds and 33% of finishing herds (Mortensen et al., 2002). To assist in the control of the disease, a modified-live PRRS vaccine derived from a Type 2 (North American) genotype virus was introduced into the country in January 1996, but by July 1996, Type 2 PRRSV was being isolated from unvaccinated herds.

Mortensen et al. (2002) undertook a retrospective case-control study of sow herds that became infected during the period June 1996 to October 1997 with the objective of identifying the likely sources of the Type 2 virus. Ultimately, 73 non-vaccinated sow herd cases were identified and matched with 129 control sow herds. Primary data sources for the statistical analyses included historical records on herd serological testing and the use of PRRSV vaccine. These data were supplemented with producer telephone interviews to collect herd management data and information regarding neighboring finishing herds' Type 2 PRRSV infection status.

The investigators determined that, with few exceptions, it was not possible to determine the source of infection and/or route of introduction. Although all 73 Type 2-infected herds were considered to have maintained a high level of biosecurity and purchased

only PRRSV-negative animals, the actual source of PRRSV was “obvious in few of the 73 case herds”, i.e., 12 herds purchased breeding stock from suppliers that had been recently infected. Interestingly, these producers had apparently not quarantined incoming stock, although they were considered to practice good biosecurity.

On the basis of the analyses, they concluded that area spread was significant when it was expressed as a formula describing distance from infected herd(s), size of neighboring herd(s), and days of exposure, although the individual factors did not necessarily support the conclusion. For example, herd size and introduction of purchased animals without quarantine were associated with infection, but herd biosecurity, and area herd and pig densities were not statistically significant factors. At odds with conventional observations, receiving semen from a PRRSV Type 2-infected boar stud was found to be protective against infection. Confronted by contradictory data, the authors seem led to conclude, “It is difficult to imagine a transmission route for PRRSV other than aerosol transmission influenced so strongly by distance to infected neighbour herds and herd size and yet unaffected by biosecurity practices.”

Experimental reports of PRRSV aerosol transmission

Experimental studies of aerosol transmission offer the means to resolve indeterminate or contradictory field information. By controlling the source of air, exposure to virus, and biosecurity (i.e. pig source, feed source, personnel, insect and vermin control) experimental transmission studies provide direct evidence of aerosol transmission and exclude indirect routes that cannot be controlled in field studies.

At the simplest level, aerosol experiments are designed such that recipient pig(s) are exposed to air from donor pig(s) intentionally infected with a specific strain of PRRSV at a known point in time. Thereafter, air from donor pigs is directed to recipient pigs through ventilation ducts connecting the two airspaces. Following a defined exposure period, recipient pigs are monitored for evidence of infection. Experiments of this type have been conducted under a variety of conditions (Table 1). Differences in experimental design include PRRSV isolate, distance between donor and recipient pigs, the presence or absence of co-infections in donor animals, environmental conditions, the age and number of donor and recipient pigs, duration of exposure, volume of air flow, day post inoculation recipient pigs were exposed, etc. Airborne transmission of PRRSV has been demonstrated under these conditions for isolates VR-2332 (Torremorell et al. 1997), ATCC VR-2402 (Wills et al. 1997b), NADC-22 (Brockmeier and Lager 2002), PRRSV-EU (Kristensen et al. 2002) and MN-184 (Cho et al. 2007) at distances of approximately 1 meter. Although studied extensively, isolate MN-30100 (Otake et al. 2002c; Trinacado et al. 2004; Fano et al. 2005) has not been transmitted by aerosols from donor to recipient pigs at distances of 1, 6, 10, or 30 meters.

Theoretically, the exposure dose of recipient pigs is dependent on the concentration of airborne infectious virus (frequency and rate of donor animal excretion) and the volume of air respired (duration of exposure and respiratory rate and tidal volume). To cause a pig to become infected by airborne transmission the infectious dose of the pig must be met or exceeded. The infectious dose may be achieved by a number of different combinations, i.e. high concentration of airborne virus and low volume of air respired, low concentration of airborne virus and high volumes of air respired. For example, 8 wk old recipient pigs

exposed to air from MN-184 infected donor pigs became infected after an exposure period of 0.25 days (Cho et al. 2007), whereas 2 wk old recipient pigs exposed to air from MN-1b infected donor pigs remained naïve even after an exposure period of 52 days (Torremorell et al. 1997). Unfortunately, estimates of airborne infectious virus per volume of air were absent from these experiments.

Excretion of airborne PRRSV

Primary replication of PRRSV occurs in nasal mucosa, specifically the alveolar macrophages in the respiratory system, and/or lymphoid tissues (Thanawongnuwech et al., 1997, 2000). Following primary replication, the virus spreads via blood (viremia) to secondary replication sites in the lung, thymus, lymph nodes, spleen, and heart (Rossow et al. 1994). Once viremic, pigs excrete virus in saliva and nasal secretions (Wills et al., 1997c; Yoon et al., 1993). Thus, aerosolization of PRRSV in respiratory exhalations, from coughing, and sneezing (Knight 1973) is biologically reasonable. Aerosols can also be generated through other means, including animal activity (Pedersen, 1993; Bonsch and Hoy, 1996) and spraying of feces and urine (Deans Ranin and Taylor 1969; Boutin et al. 1988). This is relevant since PRRSV has been recovered from urine, feces, and mammary secretions (Christopher-Hennings et al., 1995; Rossow et al., 1994; Swenson et al., 1994; Wagstrom et al., 2001; Wills et al., 1997c; Yoon et al., 1993).

Once aerosolized, detection and quantification of PRRSV requires the use of air sampling devices. Liquid air impingers are considered the most effective air samplers for the recovery of viruses (Grinshpun et al. 1996) and are widely used to collect animal viruses from aerosols (Stolze and Kaaden, 1989; Beard and Easterday, 1965; Gibson and Donaldson,

1986; Alexandersen and Donaldson, 2002; Donaldson et al., 1981; Donaldson et al., 1987; Alexandersen et al., 2002; Gillespie et al., 2000; Elazhary and Derbyshire, 1979a; Elazhary and Derbyshire, 1979b; Songer, 1967; Ehrlich and Miller, 1971; Schoenbaum et al., 1990, Moe and Harper, 1983; Larson et al., 1980; Akers et al., 1966; De Jong et al., 1974; Ijaz et al., 1994; Donaldson, 1972; Rabey et al., 1969; Donaldson and Ferris, 1975; Brown et al., 1981; Dee et al., 2005; Donaldson et al., 1983; Karim et al., 1985; Ijaz et al., 1987; Ijaz et al., 1985; De Jong et al., 1975). Attempts to recover aerosolized PRRSV from infected pigs using liquid impingers can be divided into two categories: 1) sampling environmental air from groups of pigs, 2) sampling respiratory exhalations from individual pigs (Table 2).

Sampling environmental air for detection of airborne PRRSV from groups of pigs has produced inconsistent results, depending on the air sampling device used. Air samples collected using AGI-30 impingers have yielded negative results (Torremorell et al. 1997; Otake et al. 2002; Trincado et al. 2004; Fano et al. 2005; Hermann et al. 2007). In contrast, using a high volume air sampler (HVAS) Cho et al. (2007) detected PRRSV in air samples from a group of donor pigs. The ability to capture airborne PRRSV is limited to the sensitivity of the air sampler and diagnostic assay used for detection. A number of variables are known to affect impinger sensitivity. These include impinger design (Cage et al. 1996; Doanldson et al. 1982; Jensen et al. 1992), sampling time (Lin et al. 1998), and composition of collection medium (Lin et al. 1997). Differences in flow rate (volume of air collected per unit of time) of air samplers could explain the differences in detection. The HVAS collects 400 L per min as compared to the AGI-30 impinger which samples at a rate of 12.5 L per min leading to dramatic differences in total volume of air collected. Overall, sampling environmental air for detection of airborne PRRSV from groups of pigs has produced

ambiguous results despite differences in experimental design including number of donor pigs sampled, diagnostic detection assay, location of air samplers, co-infection status, sample volume per pig, days sampled post infection, or virus isolate.

Sampling respiratory exhalations from individual pigs has also produced conflicting results. Hermann et al. (in press) collected expired air from infected (VR-2332) unanaesthetized pigs using a large canine surgical mask connected to an AGI-30 glass impinger. In addition, oral swabs were harvested to determine the presence of PRRSV in the oral cavity. Both oral swabs and collection medium was assayed by RT-PCR. Despite the presence of virus in oral fluids, air samples taken directly from acutely infected pigs were negative for presence of PRRSV.

Cho et al. (2006) collected expired air from infected (MN 30-100 and MN-184) anaesthetized pigs by placing the snout of the infected pig into a conical plastic mask connected to a collection bag. The mask and collection bag were rinsed with liquid saline and tested for presence of PRRSV. Overall, 19% of samples collected were positive and levels of PRRSV RNA were low. Although, the proportion of pigs shedding and the frequency of shedding over time varied by virus isolate, the difference in concentration of PRRSV RNA collected by isolate was not significant. According to the author the rudimentary design of the collection device may have influenced results. One possible explanation for detection of PRRSV was contamination of the sample with oral fluids, a known source of PRRSV (Prickett et al. 2007).

The source and concentration of aerosolized PRRSV may be dependent on many variables including: number and density of infected animals, stage of infection (acute vs. persistent), strain, infection with multiply agents, age, genetics, and breed of infected

animals. Failure to detect PRRSV in air samples does not rule out the presence of airborne virus. Torremorell et al. (1997) demonstrated transmission of virus from donor to recipient pigs despite negative air samples.

Stability of aerosolized PRRSV

The distance over which airborne transmission may occur is directly associated with virus stability and infectivity. Stability of aerosolized viral particles is affected by droplet size, wind direction, and speed. Infectivity of viruses in aerosols is affected by environmental factors especially UV radiation, relative humidity, and temperature (Davies and Beran 1981; Elazhary and Derbyshire 1979; Ijaz et al. 1987; Sattar et al. 1984). In general, airborne viruses are more stable at lower temperatures, but viruses are not uniformly affected by relative humidity. For example, viruses with lipoprotein envelopes tend to be more stable at lower relative humidity and non-enveloped viruses more stable at higher relative humidity (Adams et al 1982; Akers et al. 1966; De Jong et al. 1975; Donaldson and Ferris 1976; Ehrlich and Miller 1971; Elazhary and Derbyshire 1979; Larson et al. 1980; Schoenbaum et al. 1990).

Hermann et al. (2006) examined the effect of temperature and relative humidity on the infectivity of aerosolized PRRSV and estimated a half-life of 26.0 hours at -19°C and 20% relative humidity. Consistent with other arteriviruses (Donald and Ferris 1976), airborne PRRSV was more stable at lower temperatures and/or lower relative humidity. However, temperature exerted a greater effect on infectivity than relative humidity. That is, at lower temperatures the effect of different levels of relative humidity on infectivity was distinct and measurable, but at higher temperatures the effect of levels of relative humidity

on PRRSV was obscured by the rapid loss of virus infectivity. The affect of temperature on infectivity is consistent with reports of PRRSV as a fragile virus which is quickly inactivated by increased temperatures (Van Alstine et al. 1993; Pirtle and Beran 1996; Bloemraad et al. 1994; Benfield et al. 1992). Interestingly, the popular perception is that aerosolized viruses are more stable at high humidity. For example, favorable weather conditions for airborne spread of PRRSV described as relative humidity or 60% or more, low temperatures, and low wind speeds from the right direction were one of the factors attributing to the spread of PRRSV across the Netherlands (Komijn et al. 1991). Additional reports cite climate factors involved in the spread of PRRSV as low ambient temperatures, low sunlight, and high humidity favoring spread (Anon, 1992).

Infectious dose by aerosol route of exposure

The specific role aerosolized pathogens play in airborne transmission of disease has not been well characterized (Douwes et al., 2003). Airborne transmission studies generally test whether the pathogen of interest can be transmitted from donor animals to recipient animals sharing a common source of air. Using this “pigs in a box” approach fails to take into account a variety of factors that affect exposure dose recipient pigs receive, e.g., differences in excretion rates of infected donor animals (Alexandersen et al., 2003), respiratory tidal volumes of the host animal (Alexandersen et al. 2002), sedimentation and/or dilution rates of airborne particles (Morawska, 2006), rate of airborne pathogen inactivation (Hermann et al., 2007), and air sampling methods which produce inconsistent estimates of pathogen concentration (Bourgueil et al., 1992). Consequently, these transmission experiments using naturally produced aerosols are difficult to interpret or apply to the field.

In contrast, Hermann et al. (in press) described a methodology to define the relationship between exposure dose and probability of infection using artificially generated aerosols. Using PRRSV as a model to validate the infectious dose methodology, results indicated an aerosol exposure dose of $10^{3.1}$ TCID₅₀ was sufficient to cause infection in young pigs. In fact pigs exposed to infectious aerosols were easily infected e.g. 51 of 63 pigs became infected. From experimental data a dose response curve was generated. Additional experiments exposing pigs to a range of doses sufficient to encompass all sections of the dose-response curve are needed.

In conclusion, this paper provides a review of the published field and experimental data involving aerosol transmission of PRRSV. Specifically the factors influencing the likelihood of transmission including virus excretion, stability of aerosolized virus, and the infectious dose required to cause infection were critically evaluated. For aerosol transmission of PRRSV to occur a number of processes must occur sequentially. The virus must be excreted, travel to a susceptible animal, and remain infectious in concentrations sufficient to cause infection. Excretion of aerosolized PRRSV from the respiratory tract of individual pigs indicates if virus is excreted it is below the sensitivity of current air samplers (Hermann et al 2007, Trinacado et al 2004). However, aerosols generated by groups of pigs are sufficient to cause infection (Torremorell et al. 1997, Wills et al. 1997, Lager and Mengeling 2000, Brockmeier and Lager 2002, Kristensen et al. 2002). However, key facts regarding aerosol transmission of PRRSV plays in disease spread remain uncertain. Specifically, the plausibility and frequency of airborne transmission compared among possible risk factors is unclear. Similarly the probability of infection by distance has not been determined for airborne transmission of PRRSV.

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Tables and Figures

Table 1. Experimental studies on aerosol transmission of PRRSV.

Reference	Experimental Design					Environmental Conditions		Pig Characteristics		Results		
	PRRSV isolate	Meters from donors to recipient pigs	Airflow directed from donor to recipient pigs (Yes or No)	Duration of exposure (days)	Co-infected (Yes or No)	Temperature (°C)	Relative humidity (%)	No. of donor pigs and no. of recipient pigs per trial	Age (wk) or weight (kg) of donor and recipient pigs	No. of trials airborne transmission occurred	Direct contact transmission	Airborne transmission of secondary
Torremorell et al. 1997	MN-1b	1	Y	52	N	27-32	60-80	8 and 16	2 wk	0 of 1	Y	-
	VR-2332	1	Y	52	N	27-32	60-80	6 and 5	2 wk	1 of 1	Y	-
Wills et al. 1997	ATCC VR-2402	0.5	N	31	N	19-24	34-94	3 and 4	3-5 wk	1 of 3	Y	-
	ATCC VR-2402	1	N	31	N	19-24	34-94	3 and 4	3-5 wk	1 of 2	Y	-
Lager and Mengeling 2000	-	0.5	Y	7	N	-	-	3 and 3	-	1 of 2	Y	-
Brockmeier and Lager 2002	NADC-22	1	Y	7	N	-	-	3 and 3	3 wk	4 of 5	Y	-
	NADC-22	1	Y	7	Y ^b	-	-	3 and 3	3 wk	2 of 5	Y	Y
Kristensen et al. 2002	PRRSV-EU	1	Y	28	N	0.7	89	25 and 50	28 kg	1 of 1	Y	-
	PRRSV-EU	1	Y	28	N	11.0	62	26 and 43	28 kg	1 of 1	Y	-
	PRRSV-EU	1	Y	35	N	3.1	90	25 and 50	21 kg	1 of 1	Y	-
Otake et al. 2002 ^a	MN-30100	1	Y	3	N	8-11	91-95	200 and 10	20 wk	0 of 1	Y	-
		30			N					0 of 1		-
Trincado et al. 2004	MN-30100	10	Y	7	N	13-30	45-94	116 and 10	20 wk	0 of 1	Y	-
Fano et al. 2005 ^a	MN-30100	1	Y	7	Y ^c	22	67	28 and 10	8 wk ^d	0 of 1	Y	Y
		6			Y ^c					0 of 1		Y
Cho et al. 2007	MN-30100	1.3	Y	0.25	N	20	48	5 and 1	8 wk/25 kg	0 of 10	-	-
	MN-184	1.3	Y	0.25	N	20	48	5 and 1	8 wk/25 kg	4 of 10	-	-

^aRecipient pigs in two trailers were exposed to same group of donor pigs.

^bCo-infected with *Bordetella Bronchiseptica* strain KM22.

^cCo-infected with *Mycoplasma hyopneumoniae* strain 232.

^dDonor pigs were 8 wks of age and recipient pigs were 5 wks of age.

Table 2. Detection aerosolized PRRSV from infected pigs.

Reference	Experimental Design			Air Sampling Characteristics							Environmental Conditions		Results			
	PRRSV isolate	Number of donor pigs per air sample	Age (wk) / weight (kg)	Type of air sampler ^a	Collection medium ^b	Volume of collection medium (mL)	Sampling Time in minutes	Total Volume of air sampled	No. of air samples collected	Detection Assay ^c	Temperature (° C)	Relative humidity (%)	Detection of airborne PRRSV	Transmission of PRRSV to recipient pigs	Detection of secondary pathogen	Transmission of secondary pathogen to recipient pigs
Torremorell et al. 1997	MN-1b	8	2 wk	AGI-30	RPMI	-	10	125	12	VI	27-32	60-80	N	N	-	-
Torremorell et al. 1997	VR-2332	6	2 wk	AGI-30	RPMI	-	10	125	12	VI	27-32	60-80	N	Y	-	-
Cho et al. 2007	MN-30100	5	8 wk	HVAS	MEM	10	2	800	10	PCR	20	48	N	N	-	-
Cho et al. 2007	MN-184	5	8 wk	HVAS	MEM	10	2	800	10	PCR	20	48	Y	Y	-	-
Otake et al. 2002	MN-30100	200	20 wk	AGI-30	MEM	30	10	125	16	PCR/VI	8-11	91-95	N	N	-	-
Trincado et al. 2004	MN-30100	116	20 wk	AGI-30	MEM	30	10	125	84	PCR,VI,Bio	13-30	45-94	N	N	-	-
Fano et al. 2005	MN-30100	28	8 wk	AGI-30	MEM	30	10	125	14	PCR	22	67	N	N	Y ^d	Y
Hermann et al. 2007	VR-2332	1	3 wk	AGI-30 mask	PBS	20	5	62.5	140	PCR	-	-	N	-	-	-
	VR2385	1	8 wk	AGI-30 mask	PBS	20	5	65.5	42	PCR	-	-	N	-	-	-
Cho et al. 2006	MN-30100	1	8 or 24 wks	Mask device	-	-	-	-	187	PCR	-	-	Y	-	Y ^d	-
	MN-184	1	8 or 24 wks	Mask device	-	-	-	-	208	PCR	-	-	Y	-	Y ^d	-

^aAGI-30=All glass impinger 30mm (Ace Glass, Vineland, NJ), HVAS= High volume air sampler (Midwest Micro-Tek, Brookings, South Dakota, USA), AGI-30 mask = large surgical canine mask attached to AGI-30, Mask device= conical plastic mask, a breathing valve, and a collection bag flushed with saline.

^bRPMI= medium developed by Roswell Park Memorial Institute, MEM=minimum essential medium, PBS= phosphate-buffered saline

^cVI=Virus isolation, PCR=reverse transcriptase polymerase chain reaction, Bio=Bioassay

^d*Mycoplasma hyopneumoniae* strain 232.

CHAPTER 2. OPTIMIZATION OF A SAMPLING SYSTEM FOR THE RECOVERY AND DETECTION OF AIRBORNE PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND SWINE INFLUENZA VIRUS

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Abstract

The objective of this research was to optimize sampling parameters for increased recovery and detection of airborne porcine reproductive and respiratory syndrome virus (PRRSV) and swine influenza virus (SIV). Collection media containing antifoams, activated carbons, protectants, and ethylene glycol were evaluated for direct effects on factors impacting the detection of PRRSV and SIV, including virus infectivity, viability of continuous cell lines used for the isolation of these viruses, and performance of reverse-transcriptase polymerase chain reaction (RT-PCR) assays. The results showed that specific compounds influenced the likelihood of detecting PRRSV and SIV in collection medium. A subsequent study evaluated the effects of collection medium, impinger model, and sampling time on the recovery of aerosolized PRRSV using a method for making direct comparisons of up to six treatments simultaneously. The results demonstrated that various components in air sampling systems, including collection medium, impinger model, and sampling time, independently influenced the recovery and detection of PRRSV and/or SIV. Interestingly, it

was demonstrated that a 20% solution of ethylene glycol collected the greatest quantity of aerosolized PRRSV, which suggests the possibility of sampling at temperatures below freezing. Based on the results of these experiments, it is recommended that air sampling systems be optimized for the target pathogen(s) and that recovery/detection results should be interpreted in the context of the actual performance of the system.

Introduction

Airborne pathogens are detected by recovering the target microorganism in a collection medium (liquid, semi-solid, or solid substrate) and then assaying the substrate for the presence of the target pathogen using an appropriate microbiological assay. Various air sampling devices are available, but “impingers” are generally used to collect airborne viruses. Impingers direct a converged stream of environmental air onto a liquid collection medium to recover airborne viral particles in the liquid phase of the collection system (1, 10, 12, 14, 16, 17). Impingers are generally considered more effective for capturing airborne viruses than filters, bubblers, or impactors (18, 19, 20).

A number of variables are known to affect impinger collection efficiency. These include impinger design (5, 13, 24), sampling time (28), and composition of collection medium (31). In addition, specific compounds are sometimes added to impinger collection medium to preserve virus infectivity during the collection process (37, 38, 40).

This research focused on specific aspects of optimizing the collection and detection of aerosolized porcine reproductive and respiratory syndrome virus (PRRSV) and swine influenza virus (SIV) in air sampling systems. The first study focused on virus detection. Compounds added to collection media to enhance collection efficiency (i.e., antifoams,

bovine serum albumin, gelatin, mucin, activated carbon, and ethylene glycol) were evaluated for direct effects on virus infectivity, on the viability of continuous cell lines used for the isolation of these viruses, and on the performance of reverse-transcriptase polymerase chain reaction (RT-PCR) assays. The second study focused on optimizing sampling parameters for the collection of PRRSV from aerosols, including media composition, impinger model, and sampling time.

Materials and Methods

Porcine reproductive respiratory syndrome virus. The North American prototype PRRSV ATCC VR-2332 (American Type Culture Collection, Manassas, VA) was used in this study. The complete virus genomic sequence has been published (GenBank accession number PRU87392). The virus was propagated on MARC-145 cells, a clone of the African monkey kidney cell line MA-104 that is considered highly permissive to PRRSV (27).

Swine influenza virus. A field isolate of SIV designated A/Swine/Iowa/73 (H₁N₁) (National Veterinary Service Laboratories, Ames, IA) was used in this study. The virus was propagated on Madin Darby Canine Kidney (MDCK) cells.

Cell lines. Virus propagation, microinfectivity assays, and neutral red (NR) cell viability assays were performed on MARC-145 and MDCK (American Type Culture Collection, Manassas, VA) continuous cell lines. Cells were propagated and maintained in 75cm² flasks (Corning, 3150, Corning, NY). Growth medium for both cell lines consisted of minimal essential medium (MEM) (Sigma Chemical Co., M4655, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Sigma, F4922, St. Louis, MO), 50 µg of gentamicin (Sigma, G1272, St. Louis, MO) per ml, 0.25 µg of amphotericin B (Fungizone;

Sigma, A2942, St. Louis, MO) per ml, and 100 µg of penicillin-streptomycin (Sigma, P0781, St. Louis, MO) per ml.

Virus titration. Samples were titrated following the protocols described below. Virus titers were calculated using the Spearman-Kärber method (22) and expressed as 50% tissue culture infection dose 50 (TCID₅₀) per ml.

(i) PRRSV. For PRRSV, 200 µl of MARC-145 cells suspended in MEM growth medium at a concentration of 4×10^5 cells per ml were added to each well of a 96-well plate (Corning®, 3596, Corning, NY). Plates were incubated at 37°C in a humidified 5% CO₂ incubator until the cell monolayer was confluent. Samples were serially 10-fold diluted (10^0 to 10^{-5}) in MEM. Growth medium was discarded and four wells were inoculated with 100 µl of sample at each dilution. After incubating for 2 hr, the inoculum was discarded and 200 µl of growth medium with reduced FBS (5%) was added to each well. Plates were incubated at 37°C in a humidified 5% CO₂ incubator for 48 hrs. Following incubation, cells were fixed with aqueous 80% acetone solution and stained with a fluorescein isothiocyanate-conjugated monoclonal antibody specific for PRRSV (SDOW17, Rural Technologies, Inc., Brookings, SD). 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 duplicate and the titers were averaged.

(ii) Swine influenza virus. For SIV, 200 µl of MDCK cells suspended in MEM growth medium at a concentration of 4×10^5 cells per ml were added to each well of a 96-well plate. Plates were incubated at 37°C in a humidified 5% CO₂ incubator until cell monolayers were confluent. Treatment samples were serially 10-fold diluted (10^0 to 10^{-5}) in inoculation medium. Inoculation medium consisted of MEM supplemented with 5% (v/v)

bovine serum albumin (BSA) (Invitrogen, 15260-037, Carlsbad, CA) 50 µg of gentamicin per ml, 0.25 µg of amphotericin B per ml, 100 µl per ml of 200 mM L-glutamine, and TPCK-treated trypsin (Worthington Biochemical, LS003740, Lakewood, NJ) at 4 µg per ml. Growth medium was discarded and cells were rinsed 3 times with MEM containing TPCK-treated trypsin at 4 µg per ml. Four wells were inoculated with 100 µl of sample at each dilution and then plates were incubated at 37°C in a humidified 5% CO₂ incubator. After incubating for 2 hr, the inoculum was discarded and 200 µl of inoculation medium was added to each well. Plates were then incubated at 37°C in a humidified 5% CO₂ incubator for 3 to 6 d. Cells examined for cytopathic effects (CPE) daily. At d 6, or after the appearance of CPE, plates were fixed and stained as previously described by Clavijo et al. (8). Presence of SIV in cells was confirmed by an immunoperoxidase assay using a monoclonal antibody specific for influenza A virus nucleoprotein. Virus titers were determined by the number of wells at each dilution showing CPE and/or a positive reaction. Each sample was run in duplicate and the titers averaged.

Polymerase chain reaction. PRRSV and SIV RNA for real-time reverse transcriptase polymerase chain reaction (RT-PCR) amplification was extracted from 0.14 ml of sample with a QIAamp viral RNA minikit (Qiagen Inc., 210210, Valencia, CA) following the protocols recommended by the manufacturer. Real-time RT-PCR quantification was performed using an ABI Prism® 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). Primers specific for ORF7 (PRRSV) and NA segment (SIV) were synthesized by Integrated DNA Technologies (Coralville, IA) and MGB probes were synthesized by Applied Biosystems (Foster City, CA). The thermal profile for amplification of both PRRS and SIV viral RNA was a reverse transcription at 50°C for 30 min, followed

by enzyme activation at 95°C for 15 min, then 40 cycles of denaturation at 94°C for 15 s and a combined annealing/extension step at 60°C for 60 s. Fluorescence data capture occurred at the combined annealing/extension stage. For each assay, a standard curve was generated using standards (10^1 to 10^6 TCID₅₀ equivalents per ml) and positive and negative control samples were tested with the unknowns. The unit expression for RT-PCR for PRRSV is TCID₅₀/ml which represents quantity of total viral RNA in samples relative to standards in which the amount of measurable infectious viruses was quantified using microtitration infectivity assays. Quantitative RT-PCR values are estimates of total viral RNA present in samples including both infectious and inactivated virus.

Experiment One: Effects of compounds on cell viability, virus infectivity, and polymerase chain reaction performance. Specific compounds sometimes added to air sampling collection media to improve collection efficiency were evaluated for direct effects on cell viability, virus infectivity, and quantitative RT-PCR assays specific for PRRSV and SIV.

Media compounds. Compounds tested included antifoams, protectants (BSA, gelatin, mucin), sorbents (activated carbon), and ethylene glycol. Effects on cell viability were assessed by exposing MARC-145 and MDCK cells to these compounds for 2 h and then measuring differences between treated and untreated cells using a neutral red assay. Effects on virus infectivity were measured by exposing PRRSV and SIV to these compounds for 6 h at 37°C and then comparing pre- and post-exposure titers (TCID₅₀) to non-exposed controls. Effects on the diagnostic performance of quantitative RT-PCR assays were evaluated by comparing the PCR results from virus samples with and without these

compounds. Compounds exhibiting no deleterious effects on cells, virus, or PCR assays were selected for further evaluation in Experiment Two.

(i) Antifoams. The process of impingement produces extensive foaming when the liquid collection medium contains proteins and/or carbohydrates. Antifoams are added to the collection medium to eliminate this problem (26, 37). Based on these reports, six antifoams (Sigma, St. Louis, MO), i.e., Antifoam 204 (A6426), Antifoam A Emulsion (A5758), Antifoam B Emulsion (A5757), Antifoam C Emulsion (A8011), Antifoam O-30 (A8082), and Antifoam SE-15 (A8582) were evaluated. Although their exact composition is proprietary information, this selection included both organic (Antifoam 204, Antifoam O-30) and silicone-based (Antifoam A, Emulsion, Antifoam B Emulsion, Antifoam C Emulsion, and Antifoam SE-15) antifoams. Antifoams were diluted in PBS (1X) to 0.01% (v/v) and tested for their effects on cell viability (neutral red assay), virus infectivity (TCID₅₀), and diagnostic performance (RT-PCR).

(ii) Protectants. The addition of BSA (38), mucin (35), and/or gelatin (38) to collection or suspension medium has been shown to reduce the rate of virus inactivation and preserve virus infectivity during the process of impingement or aerosolization. Based on these reports, solutions of BSA (A9418), gelatin (G1890), and mucin (M1788) (Sigma, St. Louis, MO) at a concentration of 1.0% (w/v) in PBS (1X) were examined for effects on cell viability (neutral red assay), virus infectivity (TCID₅₀), and diagnostic performance (RT-PCR).

(iii) Sorbents. Activated carbon adsorbs viruses (3, 9) and may enhance infectivity (7). Based on these reports, one peat based (Sigma, C9157, St. Louis, MO) and one wood based (Cal-Pacific, Fields Landing, CA) activated carbon products were tested at five

treatment levels (0.2, 1.0, 2.0, 10.0, 20.0% (w/v)) in PBS (1X) for effects on cell viability (neutral red assay). Additionally, activated carbon (1.0%) was examined for effects on diagnostic performance (RT-PCR).

(iv) Ethylene glycol. Although its use in collection media has not been described previously, ethylene glycol was evaluated because it offers the possibility of sampling at temperatures below 0°C (32°F), i.e., ethylene glycol freezing point is -13°C. A 20% solution of ethylene glycol (Sigma, 29,323-7, St. Louis, MO) (v/v) in PBS (1X) was tested for effects on cell viability (neutral red assay), virus infectivity (TCID₅₀), and diagnostic performance (RT-PCR). A 20% solution was selected to decrease the medium's freezing point to -8° C.

Neutral red cell viability assay. A neutral red (NR) cell viability assay was used to quantify the direct effects of compounds on MARC-145 and MDCK cells (2). The quantity of dye taken up by cells was estimated using a spectrophotometer and then the effect of each treatment was determined by comparing the absorbance value (cell viability) of treated wells to the untreated control wells.

In brief, the NR assay was performed by adding 200 µl of each compound at the concentrations to be tested [antifoams (0.1%), ethylene glycol (20%), protectants (1.0%)] to 12 wells of a 96-well microtitration plate containing a 75% confluent monolayer of MARC-145 or MDCK cells. Each compound and concentration was tested eight times (8 plates). To account for plate-to-plate variability, all within-group treatments (antifoams, ethylene glycol, protectants) and untreated controls (12 wells) were present on all plates. Cells were exposed to compounds for 2 h at 37°C in a 5% CO₂ humidified incubator. Following exposure, compounds were discarded and replaced with maintenance medium. Plates were incubated for an additional 24 h at 37°C in a 5% CO₂ humidified incubator. Following incubation, the

medium was replaced with filtered (Millipore, Super-Q, ZFSQ115P4, cartridges, CDMB01204, CDAC01204, CP2001003, PMEG09002, Millipore, Billerica, MA, USA), sterilized water containing 40 µg per ml of neutral red (Sigma Chemical Co., N 2889, St. Louis, MO). Plates were incubated for 3 h at 37°C in a 5% CO₂ humidified incubator to allow for uptake of the dye. To remove the dye not taken up by living cells, the wells were rinsed with a sterile water solution containing 0.5% formaldehyde and 1% CaCl₂. To extract the dye taken up by viable cells, 200 µl of 1% acetic acid and 50% ethanol in sterile water was added to each well. The plate was left to stand at ambient temperature for 5 min and then agitated on a microplate shaker for 30 min to ensure that the dye had been released from the cells and mixed into solution. The reaction was quantified using a spectrophotometer (Bio-Tek Instruments Inc., ELx800, Winooski, VT) at a wavelength of 540 nm and the results were reported as mean absorbance.

Virus infectivity and RT-PCR detection. One ml aliquots of stock virus (PRRSV and SIV) were added to 50 ml of collection media (PBS) containing the compounds and concentrations to be tested [antifoams (0.1%), ethylene glycol (20%), BSA (1.0%), gelatin (1.0%), and mucin (1.0%)] in 250 ml media bottles. Following the addition of virus, the collection media compounds were incubated on a stir plate for 6 hr at 37°C in a 5% CO₂ humidified incubator. Samples were taken at 0 and 6 hr, aliquoted, and stored frozen at -80°C. Each compound and concentration was tested three times. Microtitration infectivity assays and quantitative RT-PCRs were performed on all samples (concentrations x replicates) concurrently.

Experiment Two: Effect of collection media, impinger, and sampling time on collection of aerosolized PRRSV. The objective of Experiment Two was to optimize

aerosol sampling parameters for collection of PRRSV. Collection medium (compounds selected for further evaluation from Experiment One), impinger model, and sampling time were evaluated relative to recovery of aerosolized PRRSV.

Aerosolization of PRRSV. PRRSV diluted in PBS (1X) to a titer of $1 \times 10^{6.33}$ TCID₅₀ was aerosolized using a 24-jet Collison nebulizer (BGI Inc., CN60, Waltham, MA) operated on compressed air (Sears Roebuck, 00916734000, Hoffman Estates, IL) at 40 P.S.I. producing 80 liters of free air per minute. The aerosolized PRRSV flowed into a 5 gallon glass reservoir (The Home Brewery Inc., B26, Ozark, MO) modified to allow simultaneous sample collection at 6 outlet ports (Figure 1). Outlet ports were installed by drilling six equidistantly spaced holes at the circumference of the glass reservoir and permanently attaching a glass stem (0.5 in ID, x 1.5 in) to each hole. Clear tubing (0.375 in ID, 0.125 in wall thickness) (Fisher Scientific, 14-169-7H, Hampton, NH) was used to connect the impingers to the outlet ports (Figure 1). This arrangement made it possible to test up to six different treatments simultaneously on the same cloud of aerosolized PRRSV.

The concentration of PRRS viral RNA in the suspension fluid was monitored during operation of the nebulizer. Samples were collected by inserting plastic tubing (0.050 ID, x 0.020 in) (Fisher Scientific, 14-170-15E, Hampton, NH) through the nozzle of the nebulizer and into the virus solution. One ml samples of the virus solution were collected at 5 min intervals using a syringe (Fisher Scientific, 14-823-69, Hampton, NH) and hypodermic needle (Fisher Scientific, NC9062128, Hampton, NH) inserted into the plastic tubing. Samples were assayed by quantitative RT-PCR.

Sampling of aerosolized PRRSV.

(i) Collection medium. Six collection medium treatments were compared on the basis of recovery of aerosolized PRRSV from the reservoir. PBS (1X) was used as the diluent in all treatments. Medium treatments were: 1) PBS, 2) PBS and 1% activated carbon (Cal-Pacific, Fields Landing, CA) (w/v), 3) PBS and 0.5% BSA (w/v), 4) PBS and 20% ethylene glycol (v/v), 5) PBS, 0.5% BSA, and 1% activated carbon, 6) PBS, 20% ethylene glycol, 0.5% BSA, and 1% activated carbon (Table 1).

(ii) Impingers. Three impinger models [AGI-30 (Ace Glass, 7540-10, Vineland, NJ), AGI-4 (6 liter) (Ace Glass, 7541-10, Vineland, NJ), and SKC BioSampler® (SCK Inc., 225-9595, Eighty Four, PA)] were compared in terms of recovery of aerosolized PRRSV. At a vacuum pressure of ≤ -0.05 ATM, the AGI-30 and SKC BioSampler® operated at a flow rate of 12.5 liters per minute (L per min) and the AGI-4 (6 liter) operated at 6.0 L per min. Vacuum pressure was maintained using oil-less pumps (Fisher, S413801, Hampton, NH) and was monitored using a vacuum pressure gauge (Cato Western Inc., G-S4LM20-VAC-100, Tucson, AZ). Flow rates of impingers in L per min were verified using a flow meter (Dwyer Instruments Inc., DW-806, Michigan City, IN).

(iii) Sampling time. The effect of sampling time (0, 1, 2, 5, 10, 15, and 20 min) on the collection of aerosolized PRRSV was evaluated by medium treatment ($n = 6$) and impinger model ($n = 3$). For each replicate, each of six impingers of the identical model was filled with 20 ml of one of the six collection media treatments to be tested. All six impingers sampled the same aerosol cloud for the designated sampling time, after which the collection fluid was harvested, aliquoted, and stored at -80° C. The experiment was repeated until each sampling time had been examined. The model was replicated three times for each of the three impinger models. A total of 63 experimental runs were completed with the six

collection medium treatments (7 time points \times 3 impinger models \times 3 replications).

Recovery of PRRSV was determined by quantitative RT-PCR. To reduce variability, RT-PCR was performed on the collection media samples concurrently.

Statistical analysis. Media compound treatments evaluated in Experiment One were compared by analysis of variance (ANOVA) (JMP®, SAS Institute Inc., Cary, NC, USA) using the data from the neutral red cell viability assays, microinfectivity assays, and quantitative RT-PCR assays. Results were reported as least square means. The null hypothesis stated that the means of the treatments and the means of the controls were equal. A significance level of <0.05 was used as the minimum acceptable p -value. If the means were significantly different, individual pair-wise treatment comparisons were performed using Student's t -test.

Quantitative RT-PCR data from collection media treatments in Experiment Two were analyzed using repeated measures MANOVA (JMP®, SAS Institute Inc., Cary, NC, USA) using sampling time as the repeating variable. The model included the main effects of impinger and collection media. Two-way and three-way interactions, i.e., impinger \times time, collection media \times time, impinger \times collection media, and impinger \times collection media \times time, were included in the model. Results were reported as least square means. A significance level of <0.05 was required as the minimum acceptable p -value. If the MANOVA was significant, one-way ANOVAs were performed at each time point. If the ANOVA was significant, individual pair-wise comparisons was performed at that time point using Student's t -test.

Results

Experiment One: Effects of media compounds on cell viability, virus infectivity, and polymerase chain reaction performance.

Antifoams. Two antifoams (A Emulsion, C Emulsion) of the six antifoams evaluated had no detrimental effect on the viability of either MARC-145 or MDCK cells (Table 2). That is, their NR assay absorbance values were not significantly different from the untreated control absorbance values ($p > 0.05$). The four remaining antifoams had absorbance values that were significantly lower than the untreated controls for MARC-145 and/or MDCK cells ($p < 0.05$), suggesting that these antifoams adversely affected one or both of the cell lines at the concentrations tested. When compared to controls, exposure of PRRSV and SIV to antifoams did not significantly affect the titers of infectious virus ($TCID_{50}$) or quantitative RT-PCR results (Table 3). On the basis of the overall results, antifoam A Emulsion (0.01%) was selected for use in Experiment Two.

Protectants. Solutions of BSA, gelatin, and mucin were tested for effects on the cell viability of MARC-145 and MDCK cells. Exposure to gelatin or mucin significantly ($p < 0.05$) reduced absorbance values for both MARC-145 and MDCK cells compared to controls (Table 2). BSA significantly reduced ($p < 0.05$) the NR assay absorbance values of MDCK cells, but not MARC-145 cells. Solutions of BSA, gelatin, and mucin were also evaluated for effects on virus infectivity ($TCID_{50}$), and diagnostic performance (RT-PCR). When compared to controls, exposure to mucin significantly lowered the titer of infectious PRRSV ($p = 0.001$), but exposure to BSA or gelatin had no effect. Exposure of SIV to BSA, gelatin, or mucin had no significant effect on the titer of infectious virus. Compared to controls, quantitative RT-PCR values were significantly reduced ($p < 0.01$) for PRRSV with the

addition of gelatin and for SIV with the addition of mucin. On the basis of the overall results, BSA (1%) was selected for use in Experiment Two.

Sorbents. Activated carbon was cytotoxic to cell lines at all concentrations tested. Therefore, it was only possible to test the direct effect of activated carbon on diagnostic performance (RT-PCR). The addition of a wood-based activated carbon product (Cal-Pacific, Fields Landing, CA) to the collection medium had no effect on quantitative RT-PCR values for PRRSV or SIV when compared to controls ($p > 0.05$). The addition of peat-based activated carbon product (Sigma, C9157, St. Louis, MO) significantly ($p < 0.001$) reduced quantitative RT-PCR values for PRRSV and SIV, as compared to controls. On the basis of the overall results, wood-based activated carbon (1.0%) was selected for use in Experiment Two.

Ethylene glycol. Exposure of both MARC-145 and MDCK cells to a 20% ethylene glycol solution significantly reduced cell viability ($p < 0.05$) when compared to controls. The addition of ethylene glycol to collection media did not reduce the infectivity or inhibit quantitative RT-PCR for PRRSV or SIV when compared to controls. On the basis of the overall results, ethylene glycol (20%) was selected for use in Experiment Two.

Experiment Two: Effect of collection media, impinger, and sampling time on collection of aerosolized PRRSV.

Aerosolization of PRRSV. The concentration of PRRSV in the suspension fluid was measured during operation of the Collison Nebulizer by sampling at intervals then assaying the sample by quantitative RT-PCR (Figure 2). The mean titer of PRRSV in the suspension fluid across sampling times was $1 \times 10^{6.33}$ TCID₅₀. No difference was detected in the

concentration of PRRSV when comparing the initial and subsequent samples across sampling times ($p = 0.89$). These results indicated that the concentration of aerosolized PRRSV was constant over the period of aerosolization.

Sampling of aerosolized PRRSV. No interactions were detected between (impinger \times collection media \times time) ($p = 0.74$) or (collection media \times impinger) ($p = 0.20$), but a statistically significant interaction existed for both (collection media \times time) ($p = 0.0002$), and (impinger \times time) ($p = 0.002$). Therefore, the main effects of impinger and collection media were analyzed across time. The effect of impinger on PRRSV collection is presented in Figure 3. The effect of collection media on PRRSV collection is presented in Figure 4.

(i) Collection media. As estimated by quantitative RT-PCR, the mean titer (TCID₅₀ equivalents) of PRRSV in collection media treatments sampled 7 times from 0 to 20 minutes was: 1) PBS - $1 \times 10^{4.62}$, 2) PBS and 1% activated carbon - $1 \times 10^{3.93}$, 3) PBS and 0.5% BSA - $1 \times 10^{4.71}$, 4) PBS and 20% ethylene glycol - $1 \times 10^{4.80}$, 5) PBS, 0.5% BSA, and 1% activated carbon - $1 \times 10^{4.50}$, 6) PBS, 20% ethylene glycol, 0.5% BSA, and 1% activated carbon - $1 \times 10^{4.68}$. Analysis of variance of collection media treatments indicated significant differences between collection media treatments at all sampling points. Individual pair-wise comparisons of collection media treatments at each sampling time indicated that a significantly lower quantity of PRRSV was detected in the PBS + activated carbon treatment at all sampling points. PBS + ethylene glycol had the greatest quantity of recovered PRRSV at all sampling points.

(ii) Impingers. As estimated by quantitative RT-PCR, the mean titer of PRRSV collected across all time points was $1 \times 10^{4.67}$, $1 \times 10^{4.53}$, and $1 \times 10^{4.30}$ TCID₅₀ for the SCK BioSampler®, AGI-30, and AGI-4 (6 liter), respectively. The SCK BioSampler® and AGI-

30 impingers collected a significantly greater amount of PRRSV when compared to the AGI-4 (6 liter) impinger at sampling times of 10, 15, and 20 min. At sampling times of 15 and 20 min the SKC Biosampler® collected a significantly greater amount of PRRSV than the AGI-30 or AGI-4 (6-liter) (Figure 3).

(iii) Sampling time. As measured by quantitative RT-PCR, the total quantity of PRRSV collected by impingers increased as sampling time increased. The mean titer by sampling time across impinger and media was 1×10^0 , $1 \times 10^{3.66}$, $1 \times 10^{3.93}$, $1 \times 10^{4.57}$, $1 \times 10^{4.74}$, $1 \times 10^{4.96}$, and $1 \times 10^{5.17}$ TCID₅₀ for 0, 1, 2, 5, 10, 15, and 20 minutes, respectively (Figure 5).

Discussion

The probability of detecting airborne viral pathogens is dependent on three primary factors: 1) the concentration of airborne virus in the environment, 2) the ability of the air sampling system to recover airborne particles (collection efficiency), and 3) the analytical sensitivity of the diagnostic assay(s) used to detect the target pathogen in the sample. In turn, each primary factor consists of component variables. For example, component variables recognized to affect the probability of recovery and detection of an airborne viral pathogen include collection medium composition, sampler type, sampling time, sampler flow rate (15, 25, 30, 42), particle size (30), rate of reentrainment (30), and the pathogen's affinity for the collection medium (31).

Collection media described for the recovery of airborne pathogens in air impingers are varied, but include deionized water (30), buffered solutions (25), and mineral oil (31). Compounds added to collection medium to improve pathogen recovery include various

proteins (38) and antifoaming agents (6, 23, 26, 37). However, the effect of collection medium composition on the recovery and detection of airborne viruses has not been quantified in direct comparisons.

Sampling times reported for the recovery of airborne pathogens using air impingers is variable, ranging from minutes to hours (12, 28, 29, 39). Likewise, flow rates described for collection of airborne viruses are variable, ranging from 12.5 lpm (14) to 450 lpm (11). Counter-intuitively, increased sampling time and/or flow rate do not necessarily result in an increase in the quantity of pathogen recovered. For example, foot-and-mouth disease virus was detected in 21 of 21 samples at a sampling time of 30 mins, but detected in 0 of 4 samples at a sampling time of 4 hrs (33). Likewise, exotic Newcastle disease virus was detected in collection medium after two hours of air sampling, but not detected in collection medium after 8 hrs of air sampling (21). Possible explanations for a decrease in recovery and detection with longer sampling time include destruction of infectious virus particles by shear forces during the process of impingement and/or re-aerosolization (reentrainment) of captured particles. Lin et al. (31) hypothesized that the hydrophobic virus particles, i.e., enveloped viruses, may be reentrained more readily in liquid air impingers.

For the most part, the effects of component variables on the recovery of airborne viruses have not been systematically investigated. Our research examined specific component variables related to collection medium, sampling time, and impinger type in the context of the recovery and detection of PRRSV and SIV. Initially, various collection media compounds were evaluated for their effects on the detection of infectious PRRSV and SIV. Subsequently, media composition, impinger type, and sampling time were analyzed to optimize the recovery of aerosolized PRRSV.

In Experiment 1, antifoams, activated carbons, protectants, and ethylene glycol were evaluated for direct effects on the infectivity of PRRSV and SIV, on the viability of continuous cell lines used for the isolation of these viruses, and on the performance of reverse-transcriptase polymerase chain reaction (RT-PCR) assays. Each compound tested was selected for a specific property related to the collection and/or detection of PRRSV and/or SIV: 1) Antifoams are necessary to eliminate the excessive foaming that occurs during impingement when the collection medium contains proteins or carbohydrates (26, 37). 2) Activated carbon was evaluated for its potential to reduce re-entrainment in impingers by adsorbing viral particles (32, 34, 36, 41). 3) Bovine serum albumin, mucin, and gelatin have been shown to reduce the rate of virus inactivation and preserve virus infectivity during the process of impingement or aerosolization (35, 38). 4) Ethylene glycol was evaluated because it offered the possibility of collecting air samples at temperatures below 0°C.

The results of the first experiment showed that the addition of specific compounds to collection media can affect virus detection, i.e., affect viability of continuous cell lines, virus infectivity, and/or performance of RT-PCR. These effects were not necessarily uniform within a group of compounds. For example, statistically significant differences were found among antifoams in their effect on continuous cell lines, with some antifoams exhibiting no effect on cells. Cytotoxic effects were not necessarily reflected in antiviral effects. Thus, although cytotoxic to cells, antifoams, protectants, and ethylene glycol had no effect on virus infectivity. Detection of PRRSV and SIV by RT-PCR were affected by the addition of protectants and activated carbon, but not antifoams or ethylene glycol. Overall, the variable effects of collection medium compounds on the detection of PRRSV and SIV demonstrated

the need to examine each compound in the context of the compound's intended use in the air sampling system and the pathogen of interest.

Questions related to the optimization of collection media remain. In particular, additional research on the use of activated carbon or other sorbents in collection medium is warranted. In this study, the addition of a peat-based activated carbon to collection medium significantly reduced the quantity of PRRSV and SIV detected by RT-PCR. Likewise, the addition of wood-based activated carbon reduced the quantity of PRRSV detected in impinger collection medium at all time points by RT-PCR. Activated carbon is known to adsorb viruses through non-specific binding to viral surface proteins (7), but its effect on RT-PCR performance has not been described. The results in Experiments 1 and 2 suggested two possibilities: 1) activated carbons inhibited RT-PCR or 2) virus was adsorbed to carbon, but was rendered unavailable to RT-PCR assays under the conditions described in this experiment. If the latter is the case, then the possibility remains that activated carbons or other sorbents could increase collection efficiency by improving virus capture and/or reducing reentrainment by adsorption.

In Experiment 2, the media compounds selected in Experiment 1 were evaluated in the context of recovery of aerosolized PRRSV in each of three impingers (AGI-30, AGI-4 (6-liter), SKC-BioSampler®). The objective of Experiment 2 was to optimize the quantity of virus recovered by manipulating the component variables of collection media, impinger model, and sampling time. To accomplish this, a method was introduced whereby direct comparisons among up to six independent treatments were possible. The validity of the comparisons was based upon the fact that all treatments sampled the same cloud of aerosolized virus. The results from the second experiment showed that the component

variables of collection media, impinger model, and sampling time affected collection efficiency of aerosolized PRRSV. Specifically, the effects of impinger model and collection medium on recovery of PRRSV were consistent across sampling times. For example, SKC-BioSampler® and AGI-30 impinger models recovered greater quantities of aerosolized PRRSV compared to the AGI-4 (6-liter) at all sampling points. Likewise, PBS + ethylene glycol collection medium recovered the greatest quantity of PRRSV at each sampling time. As in Experiment 1, the effects of collection media, impinger model, and sampling time on the recovery of PRRSV demonstrated the necessity to examine the effects of individual air sampling variables on recovery of specific target pathogens.

Several points regarding these experiments are worth noting. First, the experiments focused on optimizing the recovery and detection of viral pathogens in aerosols. In general, this is an “under explored” area of research, despite the commonly expressed fears of airborne spread of infectious agents. Second, this study described a method for direct comparisons of up to six treatments on total viral recovery. Although this simple method has not previously been described in the literature, it offers distinct advantages in terms of experimental efficiency and statistical validity. Third, it was demonstrated that a 20% solution of ethylene glycol collected the greatest quantity of aerosolized PRRSV, which suggests possible applications in sampling at temperatures below the freezing point. A review of the literature found no previous reports of the use of ethylene glycol in aerosol collection medium.

It should be note that there are currently no standard methods for the recovery and detection of specific pathogens in aerosols. In the absence of standards, air sampling protocols must be optimized and validated for each target pathogen. Results of the recovery

and detection presence or absence of specific aerosolized pathogens made using unvalidated systems should be interpreted cautiously.

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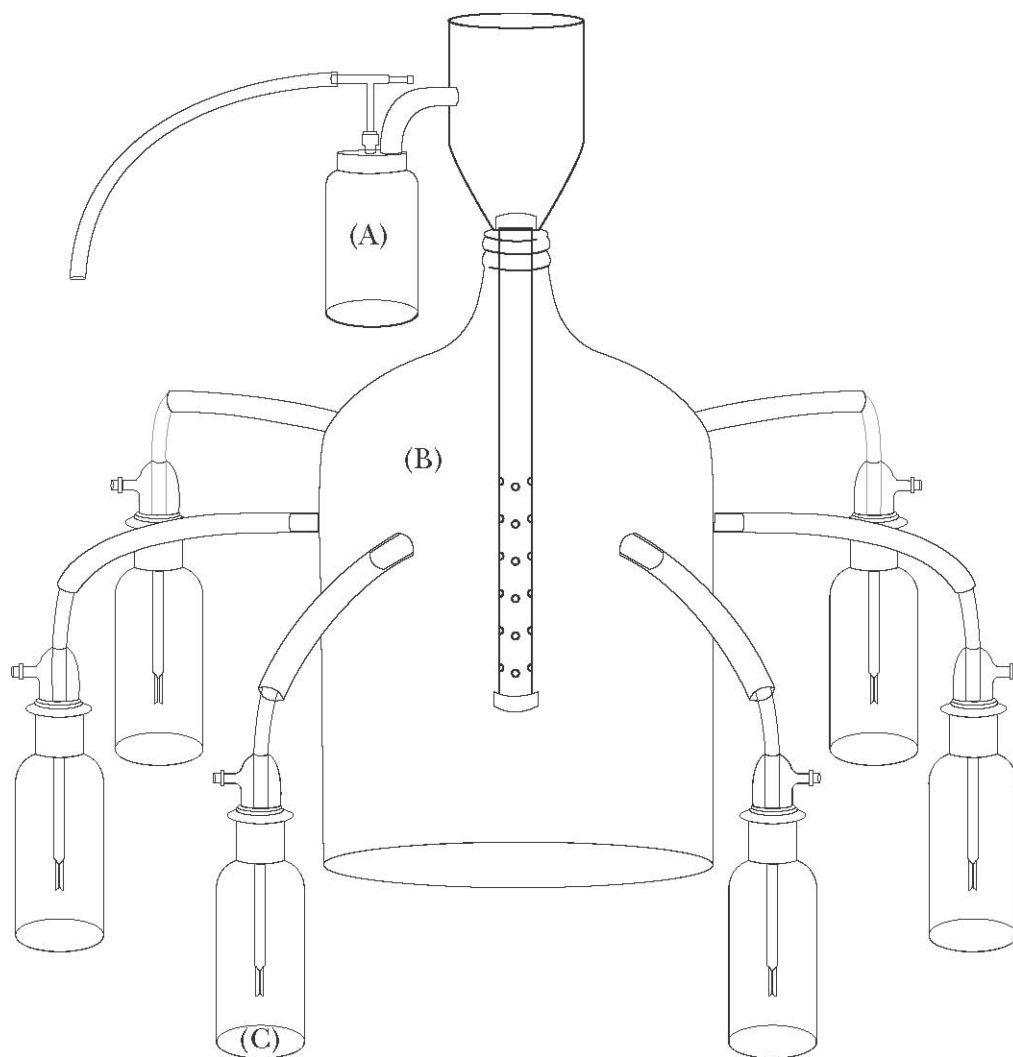
Tables and Figures

Figure 1. Illustration of the experimental design to optimize sampling time, collection media, and impingers for aerosolized PRRS virus
(A) Collision Nebulizer (B) Glass carboy (C) AGI-30 impingers.

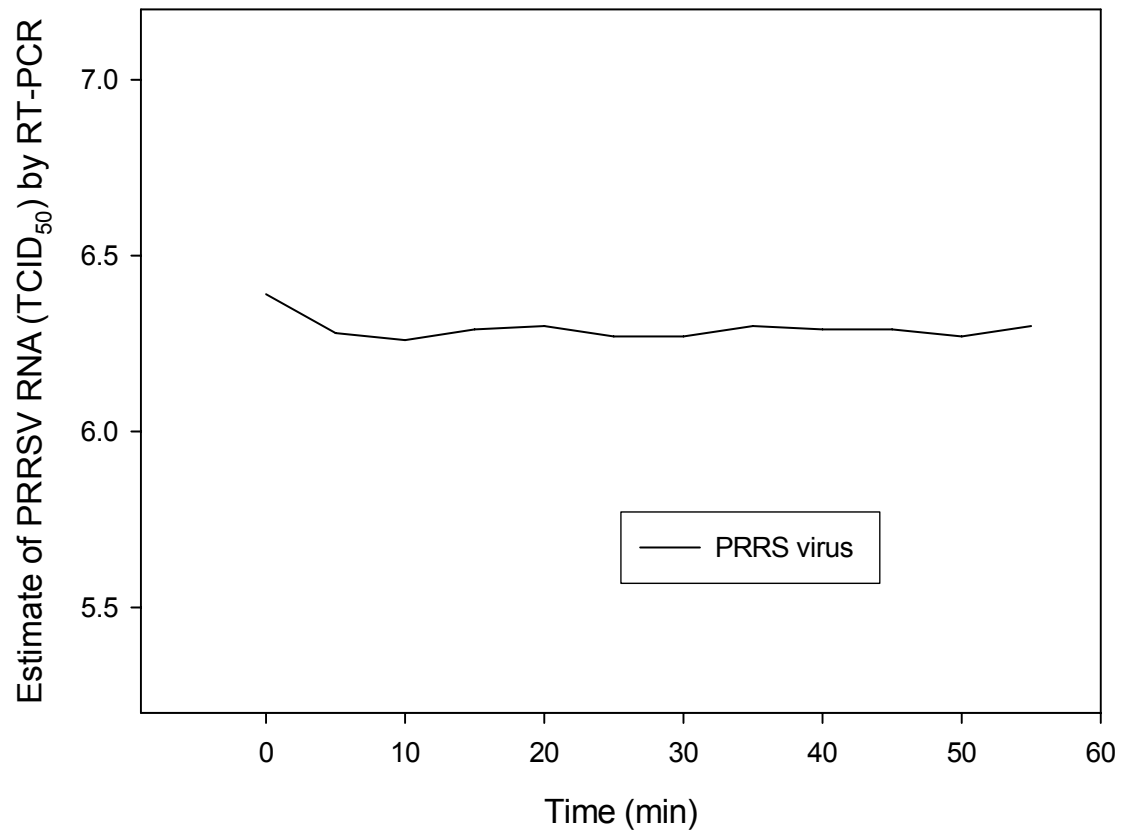


Figure 2. Quantity of PRRSV in Collision nebulizer during operation

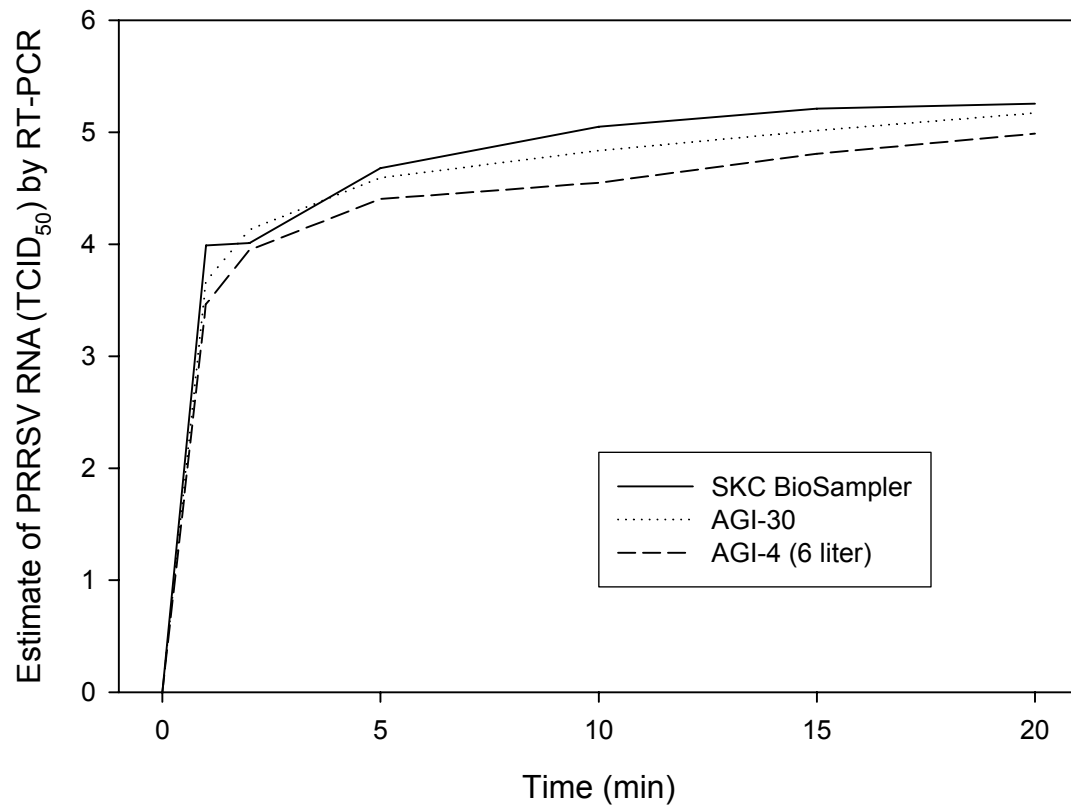


Figure 3. Collection of aerosolized PRRSV by impinger

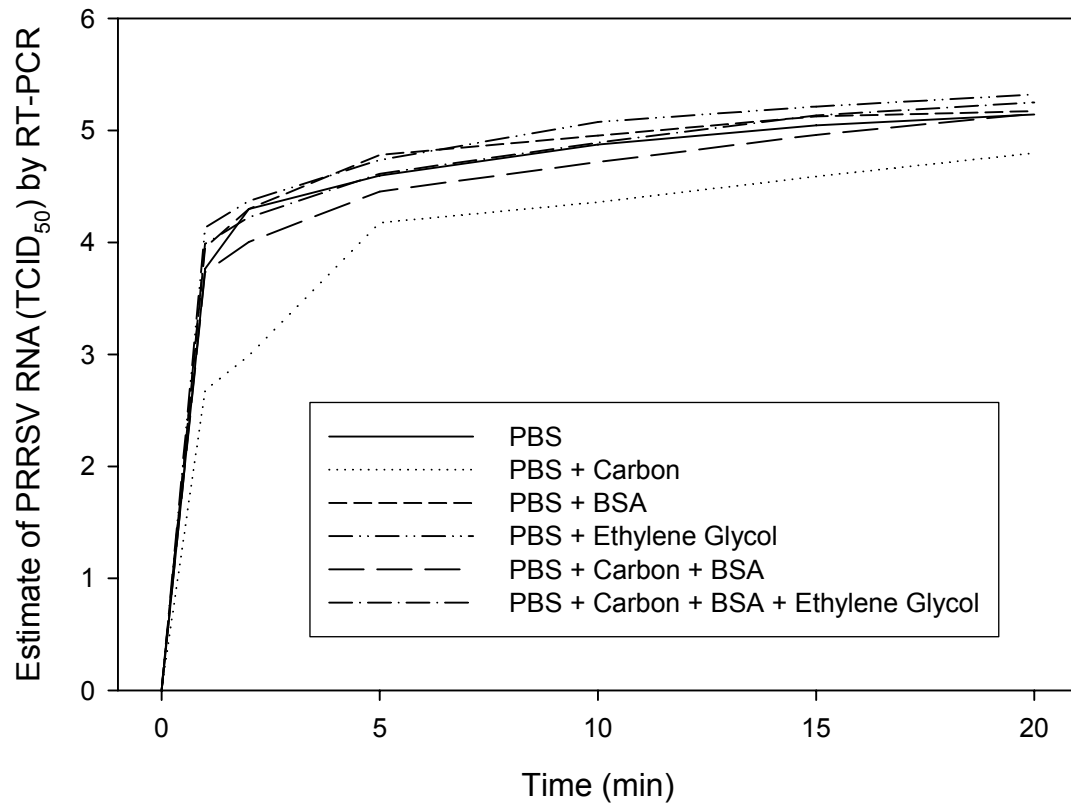


Figure 4. Collection of aerosolized PRRSV by collection media treatment

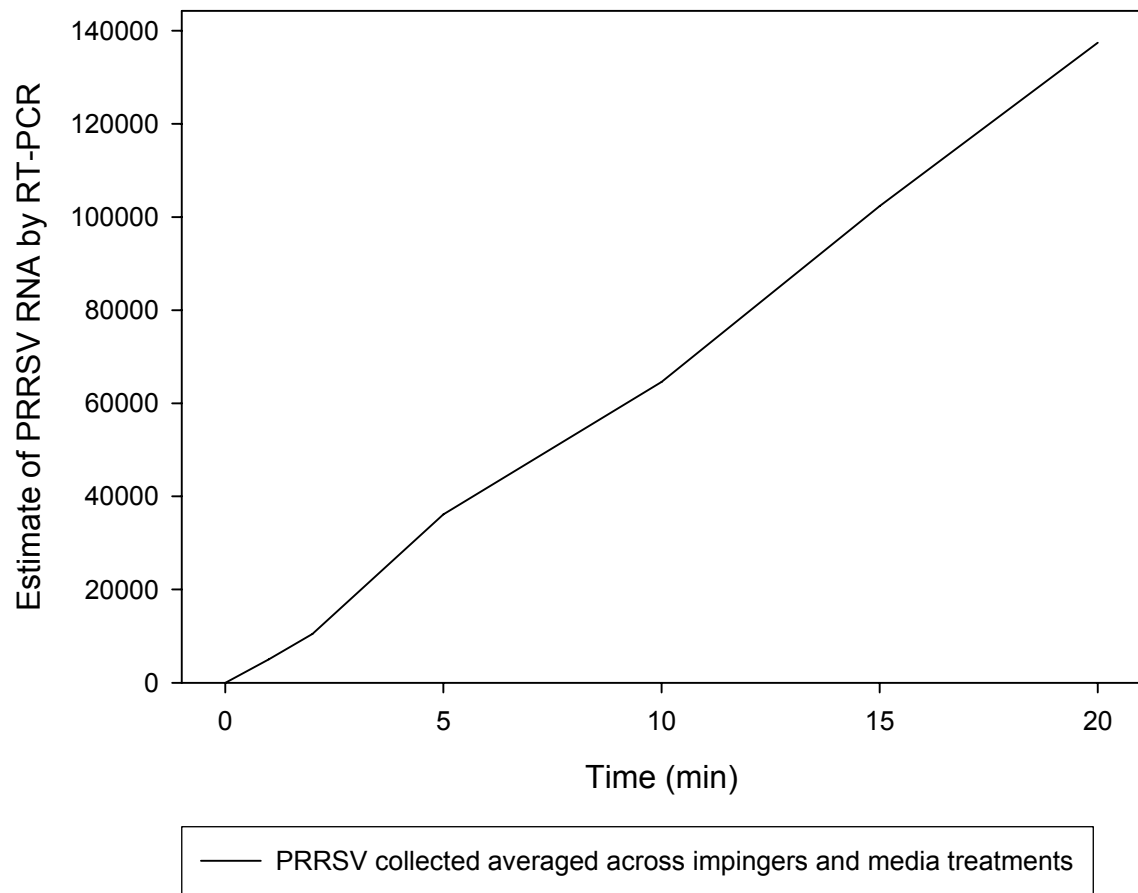


Figure 5. Aerosolized PRRSV collected over time

Table 1. Media treatments examined for collection efficiency of aerosolized PRRS virus in Experiment Two

Media Treatment	Medium Additive			
	PBS ^a	Activated carbon ^b 1%	BSA ^c 0.5%	Ethylene Glycol ^d 20%
1	+			
2	+	+		
3	+		+	
4	+			+
5	+	+		+
6	+	+	+	+

^aPhosphate buffered saline (Invitrogen, 10010-064, Carlsbad, CA)

^bActivated carbon (Cal-Pacific, Fields Landing, CA)

^cBovine serum albumin (A9418, (Sigma, St. Louis, MO).

^dEthylene glycol (29,323-7) (Sigma, St. Louis, MO).

Table 2. Univariate effects of antifoams, proteins, and ethylene glycol on viability of MARC-145 and MDCK cell lines as measured by the neutral red assay

Compound			Neutral red assay (absorbance at 540 nm)			
			MARC-145		MDCK	
Antifoams ^b	n	Concentration	Mean ^a	SEM	Mean	SEM
Control	96	0.00%	0.782 ^{wx}	0.024	0.750 ^{wx}	0.027
A Emulsion	96	0.01%	0.816 ^w	0.024	0.861 ^w	0.027
C Emulsion	96	0.01%	0.738 ^x	0.024	0.832 ^w	0.027
Antifoam 204	96	0.01%	0.641 ^y	0.024	0.729 ^{xy}	0.027
O-30	96	0.01%	0.606 ^y	0.024	0.653 ^z	0.027
B Emulsion	96	0.01%	0.588 ^y	0.024	0.598 ^{yz}	0.027
SE-15	96	0.01%	0.492 ^z	0.024	0.407 ^v	0.027
Protectants ^c and ethylene glycol ^d						
Control	96	0.00%	0.607 ^w	0.008	0.724 ^w	0.012
BSA	96	1.00%	0.597 ^w	0.008	0.592 ^y	0.012
Mucin	96	1.00%	0.547 ^x	0.008	0.665 ^x	0.012
Gelatin	96	1.00%	0.502 ^y	0.008	0.646 ^x	0.012
Ethylene glycol	96	20.0%	0.505 ^y	0.008	0.603 ^y	0.012

^aValues are least square means of absorbance. Higher absorbance values indicate viable cells; lower absorbance values indicate cell damage or death.

^bAntifoams: 204 (A26426), A Emulsion (A5758), B Emulsion (A5757), C Emulsion (A8011), O-30 (A8082), and SE-15 (A8582) (Sigma, St. Louis, MO).

^cProtectants: bovine serum albumin (A9418), gelatin (G1890), mucin (M1788) (Sigma, St. Louis, MO).

^dEthylene glycol (29,323-7) (Sigma, St. Louis, MO).

^{wxyz}Values within columns with different superscripts differ ($P < 0.01$).

Table 3. Univariate effects of antifoams, proteins, and ethylene glycol on virus infectivity and RT-PCR diagnostic performance

Compound			Virus							
			PRRSV ^f				SIV ^g			
Antifoams ^a	n	Concentration	TCID ₅₀ ^h	SEM	PCR ⁱ	SEM	TCID ₅₀	SEM	PCR	SEM
Control	3	0.00%	2.63	0.14	5.68	0.15	4.75	0.20	4.15	0.49
Antifoam 204	3	0.01%	2.70	0.14	5.60	0.15	4.40	0.20	4.16	0.49
A Emulsion	3	0.01%	2.80	0.14	5.70	0.15	4.10	0.20	2.83	0.49
B Emulsion	3	0.01%	2.80	0.14	5.76	0.15	4.40	0.20	4.33	0.49
C Emulsion	3	0.01%	2.46	0.14	5.73	0.15	4.00	0.20	4.06	0.49
O-30	3	0.01%	2.76	0.14	5.76	0.15	4.50	0.20	4.36	0.49
SE-15	3	0.01%	2.26	0.14	5.40	0.15	3.30	0.20	3.70	0.49
Protectants ^c and ethylene glycol ^d										
Control	3	0.00%	4.75 ^y	0.10	6.00 ^x	0.03	4.00	0.16	4.46 ^x	0.12
BSA	3	1.00%	4.66 ^y	0.10	6.00 ^x	0.03	4.00	0.16	4.43 ^x	0.12
Gelatin	3	1.00%	4.66 ^y	0.10	5.13 ^y	0.03	3.83	0.16	4.43 ^x	0.12
Mucin	3	1.00%	2.28 ^z	0.10	6.00 ^x	0.03	4.00	0.16	2.83 ^y	0.12
Ethylene glycol ^c	3	20.0%	5.08 ^x	0.10	6.00 ^x	0.03	4.33	0.16	4.36 ^x	0.12
Sorbents										
Control	3	0.00%	-	-	5.75 ^x	0.63	-	-	3.80 ^x	0.41
Carbon 1 ^d	3	2.00%	-	-	5.68 ^x	0.45	-	-	3.10 ^x	0.29
Carbon 2 ^e	3	2.00%	-	-	2.87 ^y	0.45	-	-	0.90 ^y	0.29

^aAntifoams: 204 (A26426), A Emulsion (A5758), B Emulsion (A5757), C Emulsion (A8011), O-30 (A8082), and SE-15 (A8582) (Sigma, St. Louis, MO)

^bProtectants: bovine serum albumin (A9418), gelatin (G1890), mucin (M1788) (Sigma, St. Louis, MO).

^cEthylene glycol (29,323-7) (Sigma, St. Louis, MO).

^dWood-based activated carbon (Cal-Pacific, Fields Landing, CA) 2% (w/v)

^ePeat-based activated carbon (Sigma, C9157, St. Louis, MO) 2% (w/v)

^fPorcine Reproductive Respiratory Syndrome Virus

^gSwine Influenza Virus

^hValues are means of tissue culture infectious dose 50% estimates calculated using the Spearman-Kärber method

ⁱValues are means of quantitative RT-PCR based on TCID₅₀ standards

^{xyz}Values within columns with different superscripts differ ($P < 0.01$).

CHAPTER 3. ANALYTICAL SENSITIVITY OF AIR SAMPLERS BASED ON UNIFORM POINT SOURCE EXPOSURE TO AIRBORNE PORCINE REPRODUCTIVE RESPIRATORY SYNDROME AND SWINE INFLUENZA VIRUSES

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Abstract

Research and surveillance activities involving airborne pathogens rely on the capture and enumeration of pathogens suspended in aerosols. The objective of this study was to estimate the analytical sensitivity (detection threshold) of each of four air samplers [(AGI-30 and AGI-4 (Ace Glass, Vineland, NJ), SKC BioSampler® (SKC Inc., Eighty Four, PA), and Midwest Micro-Tek sampler (Midwest Micro-Tek, Brookings, SD)] for porcine reproductive respiratory syndrome virus (PRRSV) and swine influenza virus (SIV). In a 5 minute sampling period under controlled conditions, the analytical sensitivity of the AGI-30, AGI-4, SKC BioSampler®, and Micro-Tek samplers for PRRSV was calculated at $1 \times 10^{1.1}$, $1 \times 10^{1.3}$, $1 \times 10^{1.1}$, and $1 \times 10^{1.2}$ TCID₅₀ equivalents per 60 liters (5 min sampling period), and for SIV, $1 \times 10^{1.4}$, $1 \times 10^{1.1}$, $1 \times 10^{1.6}$, and $1 \times 10^{1.2}$ TCID₅₀ equivalents per 60 liters (5 min sampling period), respectively. Despite marked differences in sampler design, no statistically

significant difference in analytical sensitivity was detected between samplers for collection of artificially produced aerosols containing cell culture propagated PRRSV or SIV.

Airborne transmission of viral pathogens is a significant risk to human and animal health and a challenge to disease control programs. Viruses of concern include high-risk agents such as severe acute respiratory syndrome virus (1), influenza virus (2), and foot-and-mouth disease virus (3). Airborne transmission of viruses requires release of infectious virus from a host or reservoir, transport in air, deposition in a susceptible host, entry into permissive cells, and productive infection.

Conceptually simple, airborne transmission is actually complex and dynamic. The quantity of infectious virus released from a host is affected by stage of infection, virulence of pathogen, and various host factors (4). Retention of infectivity during transport is highly variable among viruses and reflects virus-specific resistance to inactivation by environmental factors (temperature, relative humidity, solar UV radiation, etc)(5). Dispersion of airborne particles is affected by particle shape, particle size, and atmospheric conditions (wind speed, direction, topography, etc.)(6). If infectious virus reaches a susceptible host, infection is not a certainty; it is a probability function dependent upon dose, virus strain, and host-associated factors, such as gender and age (7)

Given the complexity of the process, it is understandable that aerobiology research historically has been qualitative and descriptive. However, the complex analyses needed to develop accurate models of airborne transmission for pathogens of concern require quantitative data. By definition, such an approach is based on enumeration of airborne virus

(infection and non-infectious) at various stages in the transmission process. The first step in this process is collection and enumeration of virus in air samples.

Air samplers take in environmental air and collect airborne pathogens by filtration, bubbling, or impaction. Impingement, i.e., impaction into a liquid medium, is considered the most effective approach for the recovery of viruses (8). All impingers function by directing a jet of air into a liquid collection medium, trapping viral particles therein. Aside from this common feature, impingers vary in design parameters, e.g., number, angle, and distance of the nozzle(s) relative to the liquid collection medium, flow rate, and quantity of medium in the collection reservoir. Given the variety of sampler designs, differences in sampler performance are to be expected. Therefore, the objective of this research was to compare the performance of several impingers on the basis of their analytical sensitivity, i.e., the lowest detectable quantity of airborne virus.

Four impingers were tested in the study: a) AGI-30 (Ace Glass, 7540-10, Vineland, NJ), b) AGI-4 (Ace Glass, 7541-10, Vineland, NJ), c) SKC BioSampler® (SKC Inc., 225-9595, Eighty Four, PA), and the d) Midwest Micro-Tek sampler (Midwest Micro-Tek, Brookings, SD). Impingers were compared on the basis of the quantity of virus captured from aerosol particles generated by a 6-jet Collison nebulizer (BGI Inc., CN60, Waltham, MA) in 60 liters air (5-minute sampling period). Factors that potentially affected impinger performance were held constant, e.g., collection media, sampling time, target pathogen, and the diagnostic assays used for quantification of target viruses (9).

The impingers chosen for comparison differed in design characteristics. The AGI-30 samples 12.3 to 12.6 liters of air per minute (LPM) with a single nozzle placed at 90 degrees and 30 mm from the bottom of the flask. The AGI-4 samples 6 LPM with a single nozzle

placed at a 90 degree angle and 4 mm from the flask bottom. The SKC BioSampler® operates at 12.5 LPM with three nozzles, each moving approximately 4.2 LPM of air. The nozzles are placed at an angle to the sides and bottom of the flask, thereby causing the collection liquid to swirl on the bottom and sides of the flask during operation. The Midwest Micro-Tek sampler has a self-contained internal pump operating at a sampling rate of 400 LPM. It is equipped with a fan (rotating vanes) that swirls the collection fluid in the reservoir bowl during operation.

Two viruses were used in the study: a North American isolate of porcine reproductive and respiratory syndrome virus (PRRSV) designated ATCC VR-2332 (American Type Culture Collection, Manassas, VA) and an isolate of swine influenza virus (SIV) designated A/Swine/Iowa/73 (H1N1) (National Veterinary Service Laboratories, Ames, IA). Viruses were propagated and harvested as previously described (9).

Virus concentration in the impinged samples was determined using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) assays. The PRRSV and SIV qRT-PCR protocols are described elsewhere (10). For each assay, a standard curve was generated using a series of virus standards containing PRRSV or SIV at 10^1 to 10^6 median tissue culture infectious dose 50 (TCID₅₀) per mL and sample results were reported as TCID₅₀ equivalents.

Six virus suspensions representing 6 concentrations of PRRSV and SIV were prepared. The initial virus suspension was prepared by adding 10 mL of PRRSV ($1 \times 10^{6.3}$ TCID₅₀) and 10 mL SIV ($1 \times 10^{4.6}$ TCID₅₀) to 80 mL sterile phosphate buffered saline (PBS)(pH 7.4)(Invitrogen, 10010-064, Carlsbad, CA), and 0.01% (v/v) Antifoam A Emulsion (Sigma Chemical Co., A5758, St. Louis, MO). Five serial 10-fold dilutions (10^{-1}

to 10^{-5}) were made by adding 10 mL of each subsequent dilution to 90 mL of PBS plus antifoam.

To conduct the experiment, one of the 6 virus dilutions was aerosolized into a canine anesthesia mask (SurgiVet Inc, 32393B1, Waukesha, WI) connected to an impinger with tubing (0.375 in ID, 0.125 wall thickness)(Fisher Scientific, 14-169-7H, Hampton, NH) (Figure 1). The aerosol was generated with a 6-jet Collison nebulizer (BGI Inc., CN60, Waltham, MA) operated at 20 pounds per square inch compressed air (Sears Roebuck, 00916734000, Hoffman Estates, IL). According to data generated by May (11) these conditions will produce 12 LPM of free air and aerosolize 9 mL of liquid per hour with a particle size of 2.0 μm . Four replicates were performed for each of the 4 impingers at each of the 6 virus dilutions. Air samplers were loaded with the manufacturers' recommended quantity of collection fluid (PBS) and were operated for 5 min. Suspension fluid from the nebulizer and collection fluid from the impinger were collected immediately following the 5 min sampling period and stored at -80°C until tested. All procedures were carried out within a Class II Type A2 Biological Safety Cabinet (Nuaire, 440, Plymouth, MN). Upon completion of all replicates, samples were randomized and submitted to the Iowa State University Veterinary Diagnostic Laboratory for qRT-PCR assays.

The experiment was designed to establish the relationship between the collection efficiency ($\text{quantity virus}_{\text{aerosolized}} / \text{quantity virus}_{\text{captured}}$) of each sampler across a series of dilutions. From this relationship, it was possible to estimate the analytical sensitivity for each sampler for the conditions under which the experiment was conducted. The total quantity of virus aerosolized was calculated as: (mLs of suspension fluid aerosolized by nebulizer) \times (TCID_{50} equivalents per mL). The total quantity of virus captured was

calculated as: (TCID₅₀ equivalents per mL of impinger collection fluid) × (mL of collection fluid). The total quantity of PRRSV (or SIV) aerosolized by quantity of PRRSV (or SIV) captured was analyzed by linear regression for each sampler. The y-intercept derived from this analysis was an estimate of the minimum quantity of aerosolized virus necessary to result in detection by the impingers, i.e., the analytical sensitivity of the impingers. These results were compared by analysis of variance (ANOVA) (JMP®, SAS Institute Inc., Cary, NC, USA) using the y-intercept from each replicate and reported as least square means. The null hypothesis stated that the means of the impinger analytical sensitivities were equal. A significance level of <0.05 was used as the minimum acceptable *p*-value.

The calculated analytical sensitivity of the AGI-30, AGI-4, SKC BioSampler®, and Micro-Tek samplers for PRRSV under experimental sampling parameters was $1 \times 10^{1.1}$, $1 \times 10^{1.3}$, $1 \times 10^{1.1}$, and $1 \times 10^{1.2}$ TCID₅₀ equivalents per 60 liters (5 minute sampling period), respectively (Table 1). The calculated analytical sensitivity of the AGI-30, AGI-4, SKC BioSampler®, and Micro-Tek samplers for SIV under experimental sampling parameters was $1 \times 10^{1.4}$, $1 \times 10^{1.1}$, $1 \times 10^{1.6}$, and $1 \times 10^{1.2}$ TCID₅₀ equivalents per 60 liters (5 minute sampling period), respectively (Table 1). No statistical difference in analytical sensitivity was detected among impingers for collection of PRRSV ($p = 0.88$) or SIV ($p = 0.97$) or between viruses for individual impingers, AGI-30 ($p = 0.27$), AGI-4 ($p = 0.99$), SKC BioSampler® ($p = 0.54$), and Micro-Tek ($p = 0.83$). No statistically significant difference in analytical sensitivity was detected among impingers (Figure 2) ($p = 0.23$) or between viruses (Figure 3) ($p = 0.97$) for the collapsed data.

Impingers can be characterized and compared on the basis of total collection, collection efficiency, and/or analytical sensitivity. Comparisons made on the basis of total

collection are relatively simple to perform and analyze. For example, Cage et al (12) reported that the AGI-30 impinger collected more pollen and an equivalent number of spores per cubic meter compared to a high-volume cyclonic liquid impinger (Spin-Con, Sceptor Industries, Kansas City, MO) when operated on the roof of a hospital building.

Comparisons made on the basis of total collection do not address important issues in sampler performance, e.g., efficiency. Estimates of impinger efficiency provide much more information than total collection, but present technical challenges in the design and execution of the experiment. Sampler efficiency is based on the number of target particles recovered relative to the number of target particles available per volume of air. In this study, impinger efficiency was estimated for four samplers at each of six 10-fold dilutions of PRRSV and SIV. From these results it was possible to calculate the point below which the concentration of aerosolized virus was undetectable for each impinger, i.e., the sampler's analytical sensitivity. Within the constraints of the experiment, two important conclusions can be made for the samplers and targets tested: (a) marked differences in sampler design and function parameters did not result in significant differences in collection efficiency and (b) airborne virus (infectious and non-infectious) will not be detected, if present at concentrations below the impinger's analytical sensitivity. This may provide an alternate explanation for negative results from air sampling for pathogens aerosolized in the field and under experimental conditions (13; 14; 15; 16; 17; 18). The biological significance of undetectable levels of airborne virus will depend on whether the virus is infectious and whether exposure to such a level of virus will result in productive infection in the host.

Since impinger efficiency is known to vary by the mass and size of the target, the results of this study cannot be directly extrapolated to all airborne microorganisms, i.e.,

bacteria, fungi, spores, but they are relevant to other viruses. Additionally, aerosols in this study were artificially produced and may produce different results than naturally produced aerosols, i.e. generated or dispersed by animals.

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Figures and Tables

Table 1. Analytical sensitivity of air samplers for detection of airborne porcine reproductive respiratory syndrome virus and swine influenza virus.

Impinger ^a	Flow rate (LPM ^b)	Virus ^c	Analytical Sensitivity (Log ₁₀) ^d
AGI-30	12.5	PRRSV	$1 \times 10^{1.1}$
AGI-30	12.5	SIV	$1 \times 10^{1.4}$
AGI-4	6.0	PRRSV	$1 \times 10^{1.3}$
AGI-4	6.0	SIV	$1 \times 10^{1.1}$
MicroTek	400	PRRSV	$1 \times 10^{1.2}$
MicroTek	400	SIV	$1 \times 10^{1.2}$
SKC BioSampler	12.5	PRRSV	$1 \times 10^{1.1}$
SKC BioSampler	12.5	SIV	$1 \times 10^{1.6}$

^aAGI-30 (Ace Glass, 7540-10, Vineland, NJ), AGI-4 (6 liter)(Ace Glass, 7541-10, Vineland, NJ), SKC BioSampler® (SCK Inc., 225-9595, Eighty Four, PA), and MicroTek (Midwest Micro-Tek, Brookings, SD)]

^bLiters per minute

^cPorcine reproductive respiratory syndrome virus isolate ATCC VR-2332; swine influenza virus designated A/Swine/Iowa/73 (H1N1)

^dValues are means of quantitative RT-PCR estimates based on TCID₅₀ standards.

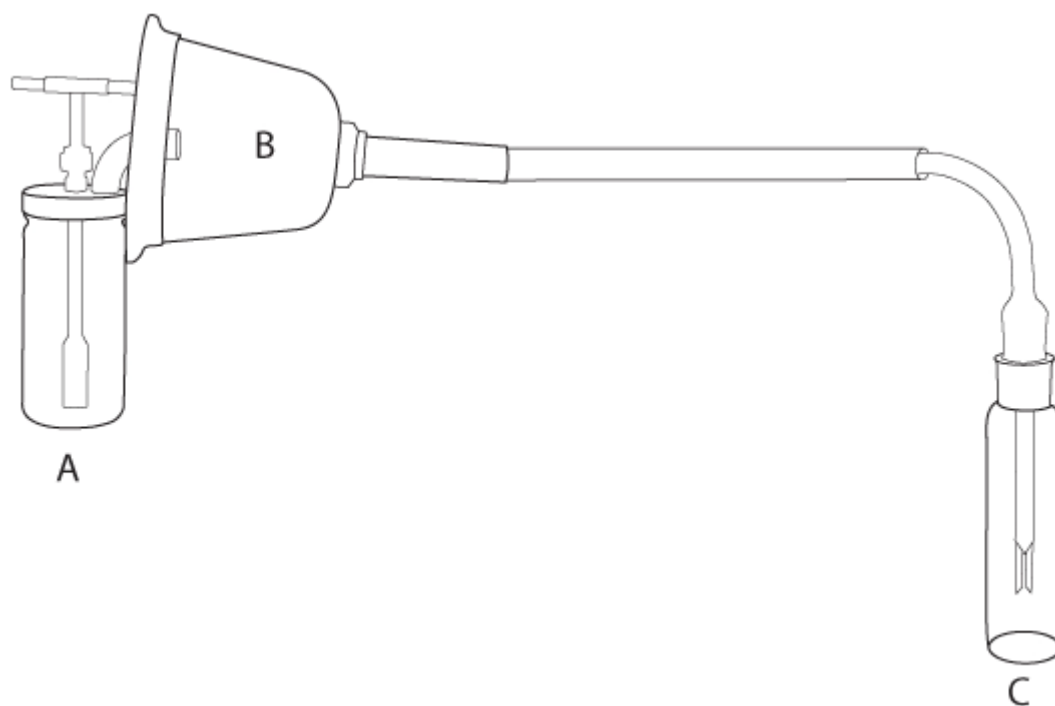


Figure 1. Experimental apparatus for estimating analytical sensitivity of air samplers (A) Collision Nebulizer (B) Canine surgical mask (C) Impinger (AGI-30 shown in illustration).

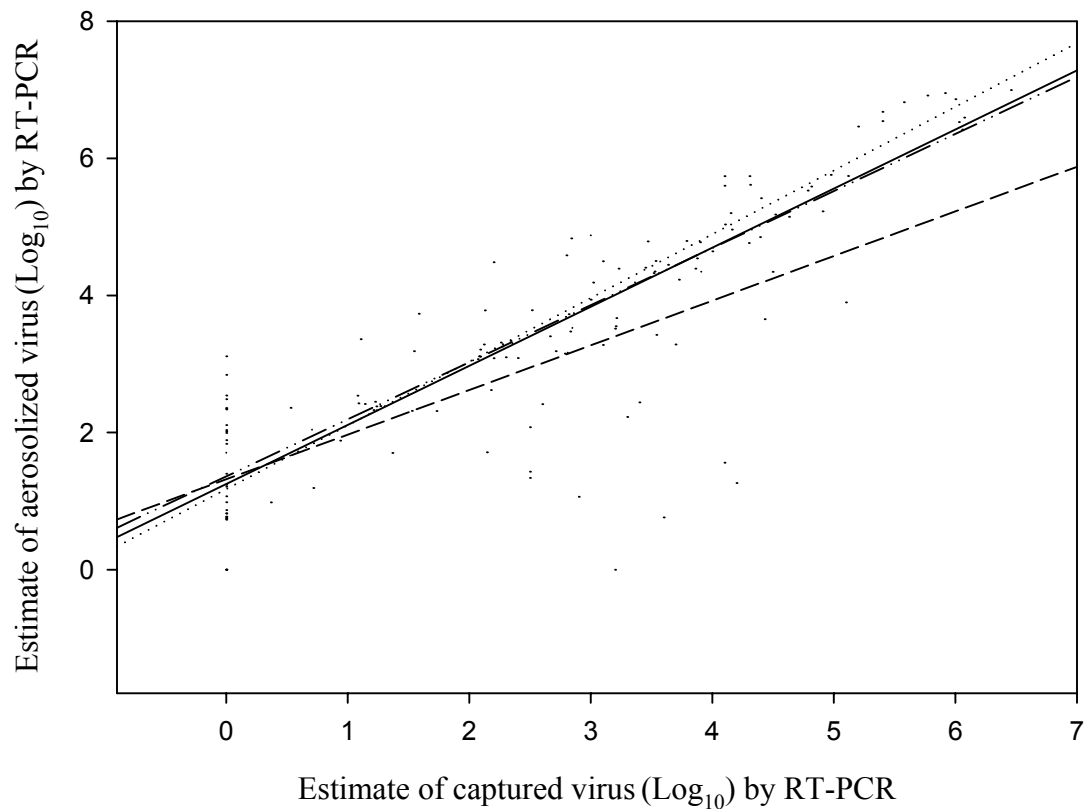


Figure 2. Determination of analytical sensitivity across viruses for AGI-30 (— · —), AGI-4 (—), SKC BioSampler® (····), and Micro-Tek (- - -) using linear regression analysis.

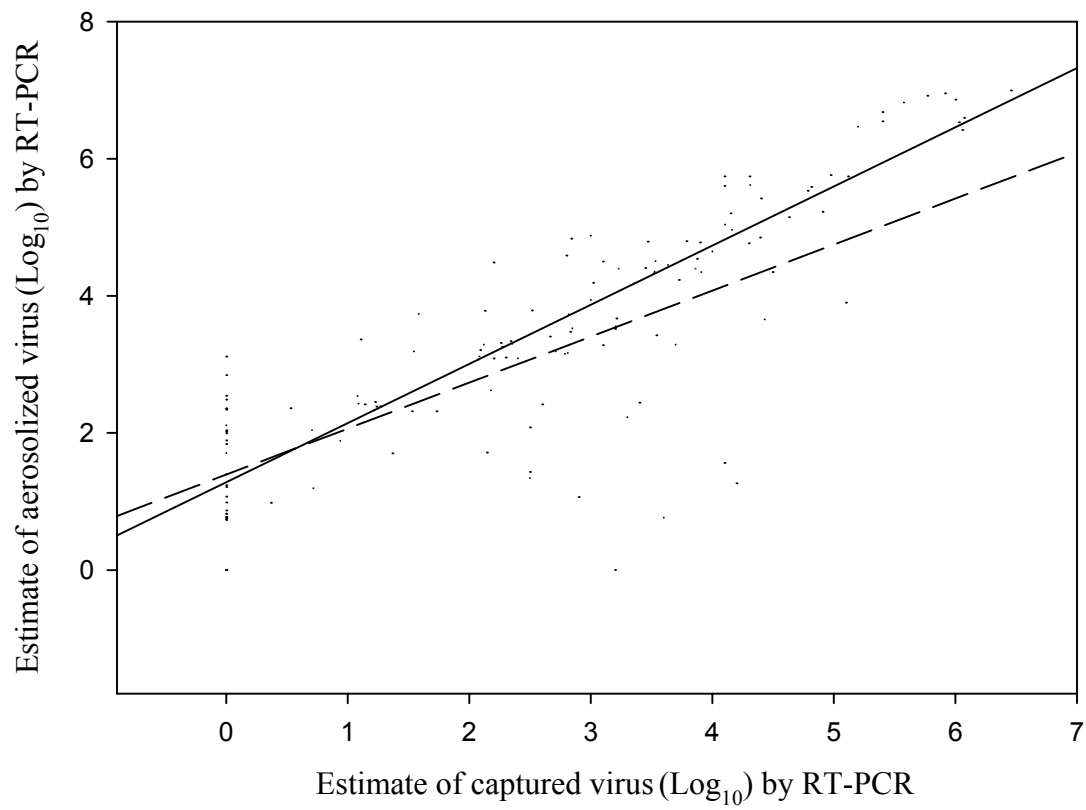


Figure 3. Determination of analytical sensitivity across impingers for PRRSV (—) and SIV (— — —) using linear regression analysis.

CHAPTER 4. DETECTION OF RESPIRATORY PATHOGENS IN AIR SAMPLES FROM ACUTELY INFECTED PIGS

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Abstract

Pathogens causing significant respiratory disease in growing pigs include porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV-2), swine influenza virus (SIV), porcine respiratory corona virus (PRCV), *Mycoplasma hyopneumoniae*, and *Bordetella bronchiseptica*. The objective of this research was to characterize the excretion of these pathogens in respirations from acutely infected pigs. Pigs were inoculated under experimental conditions with one pathogen. Samples were collected from the upper respiratory tract and respiratory exhalations. All pathogens were detected in the upper respiratory tracts of inoculated pigs, but only *M. hyopneumoniae* and *B. bronchiseptica* were detected in expired air from individually-sampled, acutely-infected pigs. These findings suggested either that acutely infected pigs did not aerosolize PRRSV, PCV-2, SIV, or PRCV, or that the quantity of virus excreted was below the analytical sensitivity (detection threshold) of current sampling and/or assay systems at the individual pig level.

Transmission from infected to susceptible animals via aerosols has been demonstrated under experimental conditions for PRRSV (1, 2), influenza virus (3), PRCV (4), *M. hyopneumoniae* (5), and *B. bronchiseptica* (1). In these studies, transmission between animals provided descriptive evidence that, under the specific conditions of the experiment, the pathogens a) were shed in respiratory exhalations of infected animals; b) remained airborne and infectious; and c) reached a susceptible animal in a dose sufficient to cause infection. Determining the quantity of pathogen aerosolized over time is a key step in the goal of understanding the parameters of aerosol transmission. Therefore, our objective was to quantify the excretion of pathogens (PRRSV, SIV, PRCV, PCV-2, *M. hyopneumoniae*, and *B. bronchiseptica*) in respiratory expirations of acutely infected pigs over time post inoculation.

Two strains of PRRSV (ATCC VR-2332 and ATCC VR-2385) (American Type Culture Collection, Manassas, VA) were used in this study. The viruses were propagated in MARC-145 cells (6). Pigs were inoculated intramuscularly with one mL of cell culture medium containing $1 \times 10^{2.0}$ 50% tissue culture infectious dose (TCID₅₀) / mL of VR-2332 PRRSV. Pigs were inoculated intranasally with 2 mL of cell culture medium containing $1 \times 10^{5.2}$ TCID₅₀ / mL of VR-2385 PRRSV. A quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) was used to detect PRRSV in impinger and swab samples (7).

Swine influenza virus [A/Swine/Minnesota/37866/1999 (H₁N₁)] (National Veterinary Service Laboratories, Ames, IA) was propagated in Madin Darby canine kidney (MDCK) cells (8). Pigs were inoculated intranasally with 3 mL of cell culture medium containing

$1 \times 10^{6.3}$ TCID₅₀ / mL of SIV. A quantitative RT-PCR was used to detect SIV in impinger and swab samples (7).

A field strain of PRCV (NADC-17) was propagated in swine testicular (ST) cells (9). Pigs were inoculated intranasally with 4 mL of cell culture medium containing 1×10^6 TCID₅₀ / mL of PRCV. A real-time multiplex RT-PCR designed to detect and differentiate transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV), was used to detect PRCV in impinger samples. Primers and MGB probes were based on nucleoprotein (N) and spike (S) protein gene sequences of TGEV or PRCV available from GenBank[®] and manufactured by Integrated DNA Technologies (Coralville, IA) and Applied Biosystems (Foster City, CA), respectively. RNA was extracted from samples using MagMax[®] Viral RNA kit (Ambion, Austin, TX) as per the manufacturer's protocol. The RT-PCR was carried out in an ABI7500 thermocycler (9600 emulation mode) with QuantiTect[®] Probe RT-PCR Kit (Qiagen, Valencia, CA) by following the protocol recommended by the manufacturer. Running conditions were as follows: 50°C for 30 minutes followed by 95°C for 30 minutes. Cycling was performed at 94°C for 15 seconds followed by 60°C for 60 seconds for a total of 40 cycles. Data acquisition was performed during the combined annealing/extension step at 60°C. Cut-off threshold cycle (Ct) for positive for each gene (S or N) was <40. Samples positive only for the N gene were considered to be positive for PRCV while samples positive for both N and S genes were considered to be positive for TGEV.

A field strain of PCV-2 (ISU-40895) was propagated on porcine kidney (PK) cells (10). Pigs were inoculated intranasally with 6 mL of cell culture medium containing $1 \times 10^{4.8}$

TCID₅₀ / mL of PCV-2. A PCR-based assay was used to detect PCV-2 in impinger and swab samples (11).

M. hyopneumoniae (strain 232), a derivative of *M. hyopneumoniae* strain 11, was propagated in Friis medium (12). Pigs were inoculated intratracheally with 10 mL of medium containing 1×10^5 color changing units (CCU) / mL. A PCR-based assay was used to detect *M. hyopneumoniae* in impinger and swab samples (13).

A field strain of *Bordetella bronchiseptica* (strain KM22) was cultured on Bordet-Gengou agar and then suspended in phosphate-buffered saline (PBS) (8). Pigs were inoculated intranasally with one mL of PBS containing 1×10^6 colony forming units of *B. bronchiseptica*. Bacterial isolation was used to detect *B. bronchiseptica* in impinger and swab samples (1).

For each pathogen, a group of pigs was inoculated under experimental conditions with one of the isolates described above. Thereafter, oral (PRRSV) or nasal (PCV-2, SIV, PRCV, *M. hyopneumoniae*, and *B. bronchiseptica*) swabs, pig respirations, and ambient room air samples were collected at regular intervals over the course of the acute phase of the infection and assayed for the presence of airborne target pathogens (Table 1).

Samples of expired air were collected from unanaesthetized pigs for five minutes. Individual pigs were physically restrained (held) and the snout placed into a large canine surgical mask (SurgiVet Inc, 32393B1, Waukesha, WI) connected to a glass impinger (AGI-30) (Ace Glass, 7540-10, Vineland, NJ) with clear tubing (Fisher Scientific, 14-169-7H, Hampton, NH) (Figure 1). Each impinger contained 20 mL of sterile PBS (pH 7.4) (1X) (Invitrogen, 10010-064, Carlsbad, CA) collection fluid. Impingers were operated at a vacuum pressure of less than half an atmosphere using oil-less pumps (Fisher Scientific,

S413801, Hampton, NH). This ensured a constant sampling flow rate of 12.5 liters per minute. Vacuum pressure was monitored constantly using a vacuum pressure gauge (Cato Western Inc., G-S4LM20-VAC-100, Tucson, AZ). To avoid negative pressure on the pig and facilitate the flow of air from the pig to the impinger, four holes (1/16 diameter) were made in the sides of the masks. After sampling, collection fluid from impingers was aliquoted into snap cap tubes (Fisher Scientific, 14-956-1B, Hampton, NH).

Nasal or oral samples were collected using sterile polyester swabs (Fisher Scientific, 14-959-90, Hampton, NH). Nasal samples were collected by inserting and rotating a swab in the nares. Oral samples were collected by dragging and rotating the swab along the gingival crevicular surface of the oral cavity. Nasal and oral swabs were immediately placed into snap cap tubes (Fisher Scientific, 14-956-1B, Hampton, NH) containing 2 mL of sterile PBS (Invitrogen, 10010-064, Carlsbad, CA).

All samples were stored frozen at -80°C until tested. Following the completion of the study, samples were completely randomized and re-labeled, and then assayed as a block for the presence of the target pathogen. A sample was considered positive if the target pathogen was detected by the specified assay (Table 2).

Swab samples (oral or nasal) demonstrated that all targeted pathogens were present at detectable levels in the upper respiratory tract (Table 3). The initial detection of agent, frequency, and proportion of positive samples in collected oral or nasal swabs varied by pathogen. All pathogens, except *M. hyopneumoniae* and PCV-2, were detected in one or more pigs from 2 to 4 DPI. In contrast, the initial detection of PCV-2 and *M. hyopneumoniae* occurred on 14 DPI. *B. bronchiseptica* had the highest proportion of positive nasal swabs

(20/20) over the course of sampling. PCV-2 had the fewest positive swab samples (3/20) over the course of sampling.

Respiratory expiration samples did not generally reflect the results of oral or nasal swab samples. *M. hyopneumoniae* was recovered from pig respiratory samples from four different pigs on 8, 17, 21, and 28 DPI. A total of 4/44 (9%) samples were positive for *M. hyopneumoniae*. *B. bronchiseptica* was recovered from expiration samples from 2 of 4 pigs on 6 DPI. A total of 2/20 (10%) aerosol samples were positive for *B. bronchiseptica*.

Respiratory samples collected from pigs infected with PRRSV, PCV-2, PRCV and SIV were negative on all sampling days (Table 3). In addition, all ambient room air samples were negative for targeted pathogens on all sampling days.

The objective of this research was to measure the level of pathogens in respiratory exhalations from acutely infected pigs. The recovery and detection of *B. bronchiseptica* and *M. hyopneumoniae* in this study was in agreement with earlier reports based on air samples collected from infected pigs (5, 14, 15). Negative results for the recovery and detection of viral pathogens were in conflict with descriptive studies reporting airborne transmission of PRRSV (1), PRCV (4), and influenza virus (3) between animals and quantitative studies reporting collection of airborne excretion of PRRSV (15). However, these results were in agreement with negative results for the detection of airborne PRRSV from infected pigs using sampling systems similar to that described in this study (5, 1). In these latter two studies, the experimental design differed in that samples were collected from the air space in which groups of pigs were housed, rather than individual pigs.

Overall, the data indicated that viral pathogens were either not present in respiratory exhalations or were below the analytical sensitivity of the sampling/detection procedures.

Specific elements that may have contributed to negative results include sample collection time, analytical sensitivity of the detection system, and isolate pathogenicity (2, 15).

Sample collection time may contribute significantly to the discordance between animal transmission studies and these results. Essentially, susceptible animals function as continuous *in vivo* monitors for the presence of airborne infectious agents; thus, optimizing the likelihood of “detection.” In contrast, *in vitro* air samples are collected for a brief interval. Maximum sample collection time is mandated by the physical design of the sampler, but prolonged impingement is generally not practical because of desiccation of sampling medium, physical destruction of targets, and/or re-entrainment of pathogens.

Impinger performance is known to be affected by a number of other factors, including collection medium composition, sampler type, sampling time, sampler flow rate (7, 16, 17), collection efficiency, and particle size (18). For PRRSV and SIV, the sampling/detection systems used in this study were previously shown capable of detecting $1 \times 10^{1.1}$ TCID₅₀ of PRRSV and $1 \times 10^{1.4}$ TCID₅₀ of SIV excreted over a five minute sampling period (19). Therefore if PRRSV and SIV were aerosolized, it was in concentrations below the impinger’s analytical sensitivity.

Overall, the results of this study confirm findings by Cho et al. (15) that airborne pathogens are generally aerosolized by individual pigs in minute quantities making detection and quantification by current sampling systems difficult. This does not preclude the need of this information. Alternate approaches, e.g., novel collection methods, sampling groups of pigs in defined air spaces, technical improvements in air samplers, may be required to successfully estimate excretion rates at the individual pig level. An important consideration of approaches that do not sample the individual pig is the underestimation of excretion rates

due to the loss of pathogens via dispersion and sedimentation. Regardless of the technical challenge, probabilistic models for within- and between-site transmission of airborne pathogens await quantitative measures of the excretion of pathogens in aerosols by pigs.

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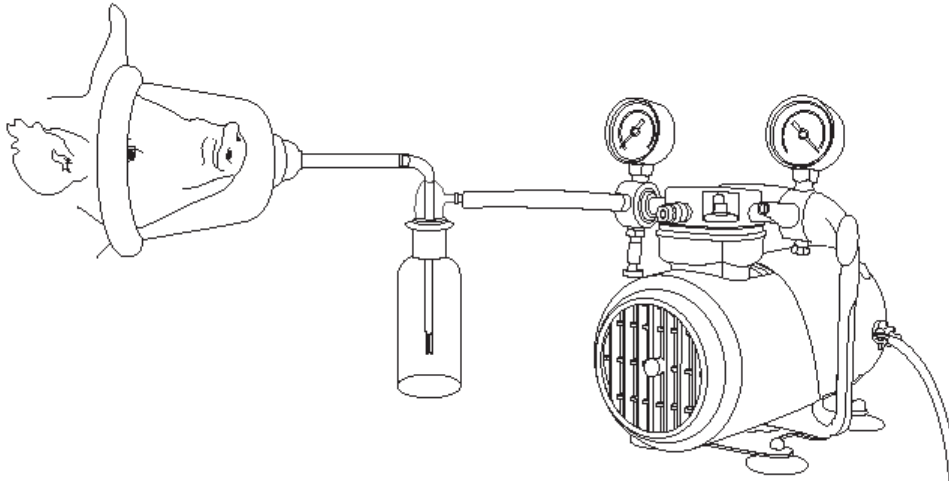
Tables and Figures

Figure 1. Diagrammatic representation of system used to collect respiratory samples from pigs

Table 1. Schedule of aerosol and swab (nasal or oral) sampling to detect pathogens excreted from inoculated pigs.

Pathogen targeted ^a	No. ^b	Age ^c	Type of sample harvested by days post inoculation ^d														
			0	1	2	4	6	8	9	11	13	14	15	17	21	23	28
PRRSV ^e	20	21	I	AE	ns	AE	AEO	AEO	ns	AEO	AEO	ns	AEO	ns	ns	ns	ns
PRRSV ^f	6	56	I	AEO	ns	AEO	AEO	AEO	ns	AEO	AEO	ns	AEO	ns	ns	ns	ns
PCV-2 ^g	4	49	I	AEN	AEN	ns	AEN	AEN	ns	ns	ns	AEN	ns	ns	ns	ns	ns
PRCV ^h	4	24	I	Ns	AEN	AEN	AEN	AEN	AEN	ns	ns	ns	ns	ns	ns	ns	ns
SIV ⁱ	4	24	I	Ns	AEN	AEN	ns	AEN	AEN	ns	ns	ns	ns	ns	ns	ns	ns
<i>M. hyopneumoniae</i> ^j	4	35	I	AEN	ns	AEN	AEN	AEN	ns	AEN	AEN	ns	AEN	AEN	AEN	AEN	AEN
<i>B. bronchiseptica</i> ^k	4	24	I	Ns	AEN	AEN	AEN	AEN	AEN	ns	ns	ns	ns	ns	ns	ns	ns

^aSamples collected from inoculated pigs were assayed for specified target pathogen.

^bNumber of individual pigs from which aerosol and swab samples were collected on each sampling day.

^cPig age in days at time of inoculation.

^dA=aerosol sample collected using all glass impingers (AGI-30), E= environmental room air sample, O= oral fluids swab, N= nasal fluid swab, ns= no sample taken.

^ePorcine reproductive and respiratory syndrome virus isolate, ATCC VR-2332

^fPorcine reproductive and respiratory syndrome virus isolate, ATCC VR-2385

^gPorcine circovirus type 2, isolate 40895

^hPorcine respiratory corona virus isolate, NADC-17

ⁱSwine influenza virus designated A/Swine/Minnesota/37866/1999 (H₁N₁)

^j*Mycoplasma hyopneumoniae* strain 232

^k*Bordetella bronchiseptica* strain KM22

Table 2. Summary of inoculated infectious agents and detection methods.

Agent ^a	Strain	Route ^b	Volume	Dose ^c	Assay ^d	Laboratory ^e	Previously described
PRRSV	VR-2332	IM	1.0 mL	10 ^{2.0} TCID ₅₀ /mL	RT-PCR	ISU	Hermann et al. 2006
PRRSV	VR-2385	IN	2.0 mL	10 ^{5.2} TCID ₅₀ /mL	RT-PCR	ISU	Hermann et al. 2006
SIV	H ₁ N ₁	IN	3.0 mL	10 ^{6.3} TCID ₅₀ /mL	RT-PCR	ISU	Hermann et al. 2006
PRCV	NADC-17	IN	4.0 mL	10 ^{6.6} TCID ₅₀ /mL	PCR	ISU	Described in methods
PCV-2	ISU-40895	IN	6.0 mL	10 ^{4.8} TCID ₅₀ /mL	PCR	SDSU	Larochelle et al. 1999
<i>M. hyopneumoniae</i>	232	IT	10 mL	10 ^{4.0} CCU/mL	PCR	SDSU	Sorensen et al. 1997
<i>B. bronchiseptica</i>	KM22	IN	1.0 mL	10 ^{6.0} CFU/mL	Isolation	NADC	Brockmeier and Lager, 2002

^aPorcine reproductive and respiratory syndrome virus isolate ATCC VR-2332; Porcine reproductive and respiratory syndrome virus isolate ATCC VR-2385; Porcine circovirus type 2 isolate 40895; Porcine respiratory coronavirus isolate NADC-17; Swine influenza virus designated A/Swine/Minnesota/37866/1999 (H₁N₁); *Mycoplasma hyopneumoniae* strain 232; *Bordetella bronchiseptica* strain KM22.

^bRoute of inoculation: IM= intramuscular, IN= intranasal, IT= intratracheal.

^cDose administered is expressed as TCID₅₀= 50% tissue culture infectious dose, CCU= color changing units, CFU= colony forming units.

^dRT-PCR= reverse transcriptase polymerase chain reaction, PCR= polymerase chain reaction, isolation= virus or bacterial isolation.

^eSamples were submitted to the following diagnostic laboratories: ISU= Iowa State University, SDSU= South Dakota State University, NADC= National Animal Disease Center.

Table 3. Results of aerosol and swab (nasal or oral) sampling to detect pathogens excreted from inoculated pigs.

Pathogen targeted ^a	No. ^b	Age ^c	Sample type ^d	Number of positive samples by days post inoculation ^e															
				0	1	2	4	6	8	9	11	13	14	15	17	21	23	28	Totals
PRRSV ^f	20	21	aerosol	I	ns	ns	ns	0	0	ns	0	0	ns	0	ns	ns	ns	ns	0/100
PRRSV ^g	6	56	aerosol	I	0	ns	0	ns	0	ns	0	0	ns	0	ns	ns	ns	ns	0/36
PCV-2 ^h	4	63	aerosol	I	0	0	ns	0	0	ns	ns	ns	0	ns	ns	ns	ns	ns	0/20
PRCV ⁱ	4	24	aerosol	I	ns	0	0	0	0	0	ns	ns	ns	ns	ns	ns	ns	ns	0/20
SIV ^j	4	24	aerosol	I	ns	0	0	ns	0	0	ns	ns	ns	ns	ns	ns	ns	ns	0/16
<i>M. hyopneumoniae</i> ^k	4	35	aerosol	I	0	ns	0	0	1	ns	0	ns	0	0	1	1	0	1	4/44
<i>B. bronchiseptica</i> ^l	4	24	aerosol	I	ns	0	0	2	0	0	ns	ns	ns	ns	ns	ns	ns	ns	2/20
PRRSV ^f	20	21	swab (oral)	I	ns	ns	ns	10	5	ns	6	6	ns	2	ns	ns	ns	ns	29/100
PRRSV ^g	6	56	swab (oral)	I	0	ns	4	ns	1	ns	2	0	ns	0	ns	ns	ns	ns	7/36
PCV-2 ^h	4	63	swab (nasal)	I	0	0	ns	0	0	ns	ns	ns	3	ns	ns	ns	ns	ns	3/20
PRCV ⁱ	4	24	swab (nasal)	I	ns	3	2	2	0	0	ns	ns	ns	ns	ns	ns	ns	ns	7/20
SIV ^j	4	24	swab (nasal)	I	ns	4	4	ns	0	0	ns	ns	ns	ns	ns	ns	ns	ns	8/16
<i>M. hyopneumoniae</i> ^k	4	35	swab (nasal)	I	0	ns	0	0	0	ns	0	ns	2	2	4	4	3	4	19/44

<i>B. bronchiseptica</i> ^l	4	24	swab (nasal)	I	ns	4	4	4	4	4	ns	ns	ns	ns	ns	ns	ns	20/20
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^aSamples collected from inoculated pigs were assayed for specified target pathogen.

^bNumber of individual pigs from which aerosol and swab samples were collected on each sampling day.

^cPig age in days at time of inoculation.

^daerosol samples collected using all glass impingers (AGI-30), oral and nasal samples collected using Dacron swab.

^eNumber of positive samples out of total samples collected, I= day of inoculation, ns=no sample taken

^fPorcine reproductive and respiratory syndrome virus isolate ATCC VR-2332

^gPorcine reproductive and respiratory syndrome virus isolate ATCC VR-2385

^hPorcine circovirus type 2 isolate 40895

ⁱPorcine respiratory corona virus isolate NADC-17

^jSwine influenza virus designated A/Swine/Minnesota/37866/1999 (H₁N₁)

^k*Mycoplasma hyopneumoniae* strain 232

^l*Bordetella bronchiseptica* strain KM22

CHAPTER 5. EFFECT OF TEMPERATURE AND RELATIVE HUMIDITY ON THE STABILITY OF INFECTIOUS PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS IN AEROSOLS

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Abstract

The objective of this experiment was to describe the stability of airborne infectious porcine reproductive and respiratory syndrome virus (PRRSV) as a function of temperature and relative humidity. A cloud of infectious PRRSV was aerosolized using 24-jet Collison nebulizer into a dynamic aerosol toroid (DAT) maintained at a specific temperature and relative humidity. The PRRSV cloud within the DAT was sampled repeatedly over time using SKC BioSampler® impingers and the total viral RNA (RT-PCR) and concentration of infectious PRRSV (TCID₅₀) in the air samples was determined. As measured by quantitative RT-PCR, PRRSV RNA was stable under the conditions evaluated in this study. Thus, a comparison of viral RNA and Rhodamine B dye, a physical tracer, found no significant difference in the slopes of the lines. Titers of infectious virus were plotted by time and the half-life (T_{1/2}) of infectious PRRSV was calculated using linear regression analysis. An

analysis of the results showed that aerosolized PRRSV was more stable at lower temperatures and/or lower relative humidity, but temperature had a greater effect on the T_{1/2} of PRRSV than relative humidity. Based on these results, an equation was derived to predict the T_{1/2} of infectious airborne PRRSV for any combination of environmental temperature and relative humidity.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped RNA virus in the family *Arteriviridae* within the order *Nidovirales* [6, 8, 32, 35]. Herds clinically affected by PRRSV infection exhibit lower farrowing rates, fewer piglets weaned, slower growth rates, lower growth efficiency, and increased mortality. The effects of PRRSV on swine production are significant and direct losses from PRRSV were estimated to cost swine producers in the United States \$560 million annually [34].

A recently emerged virus, PRRSV was first isolated at the Central Veterinary Institute (Lelystad, the Netherlands) on porcine alveolar macrophage cultures from specimens obtained from herds undergoing severe reproductive disorders [31]. Following its introduction into the domestic swine population, probably some time in the 1970's, the virus spread rapidly and at present, the herd-level prevalence of infection generally exceeds 60 percent in swine-dense regions of the world [46].

Reliable methods to prevent, control, and/or eliminate PRRSV have not been achieved, in part, because our understanding of the PRRSV transmission cycle in domestic swine is still incomplete. It is known that pigs may become infected via exposure to PRRSV by any of several routes: intranasal [18, 45], intramuscular [18, 45], oral [18, 28, 29, 42], intrauterine

[7], and vaginal [3, 17, 44]. Transmission via aerosols also occurs and was once considered the primary route of PRRSV transmission, but it has been difficult to consistently reproduce airborne transmission of PRRSV from infected to susceptible pigs under experimental conditions [4, 11, 15, 25, 26, 40, 41, 43]. Inconsistent replication of airborne transmission of PRRSV under experimental conditions suggests that we do not understand the conditions required for its occurrence. To address this lack of consistent data, we evaluated one of the stages of the process, i.e., stability of infectious PRRSV in aerosols as a function of temperature and relative humidity. Since the stability of aerosolized viruses is a function of atmospheric conditions, the objective of this experiment was to derive an equation that would predict the half-life ($T_{1/2}$) of aerosolized infectious PRRSV as a function of relative humidity and temperature. Half-life is the time in which the quantity of a substance declines by one-half [5]. $T_{1/2}$ can be calculated for any factor that decreases exponentially with time.

Material and Methods

2.1. Experimental Design

A suspension of PRRSV was aerosolized into a dynamic aerosol toroid (DAT) [16] rotating at 5 revolutions per minute and maintained at a pre-determined temperature and relative humidity. The cloud of PRRSV contained within the DAT was sampled repeatedly over time, the concentration of infectious PRRSV ($TCID_{50}$) in the samples was determined, and the $T_{1/2}$ for the specific combination of relative humidity and temperature was estimated based on the inactivation of infectious virus observed over time. A total of 18 $T_{1/2}$ estimates were

used in a regression analysis to derive an equation estimating the $T_{1/2}$ of aerosolized PRRSV for any combination of temperature and relative humidity.

2.2. Virus and cells

The North American prototype PRRSV ATCC VR-2332 (American Type Culture Collection, Manassas, VA, USA) was used in this study. The complete virus genomic sequence has been published (GenBank accession number PRU87392). Virus propagation and microinfectivity assays were performed on MARC-145 cells (National Veterinary Service Laboratory, Ames, IA, USA), a clone of the African monkey kidney cell line MA-104 that is considered highly permissive for PRRSV [23].

2.3. Virus Propagation

Virus was propagated on 24 h-old MA-104 cells prepared in 1750 cm² roller bottles (Corning, 430699, Corning, NY, USA) containing MEM growth medium: MEM (Sigma Chemical Co., M4655, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma, F4922), 50 µg/mL gentamicin (Sigma, G1272), 100 IU/mL penicillin (Sigma, G6784), 100 mg/mL streptomycin (Sigma, G6784), and 0.25 µg/mL amphotericin B (Sigma, A4888). After 24 h at 37 °C in a humidified 5% CO₂ incubator, the MEM growth medium was discarded and the bottles inoculated with 5 ml of PRRSV isolate VR-2332 at a titer of 10⁶ TCID₅₀/mL in maintenance medium: 50 mL of MEM supplemented with 2% FBS, 50 µg/mL gentamicin, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B. After 2 h at 37 °C in a humidified 5% CO₂ incubator, the inoculum was discarded and 300 mL of maintenance medium was added. Thereafter, cells were examined

for cytopathic effect (CPE) daily. When 75% CPE was observed, the medium was freeze-thawed (-80 °C / 25 °C) and cell lysates were harvested. The supernatant was centrifuged at 17 696 x g for 10 min and pooled. The supernatant harvested from the roller bottles was processed to increase virus titer using a Prep/Scale spiral wound ultrafiltration module (Model CDUF 001 LH, Millipore, Billerica, MA, USA) set to maintain a pressure of 1.40 kg/cm. Approximately 10 L of supernatant was reduced to approximately one liter of virus stock. The pooled virus stock was aliquoted into 10 mL volumes and stored at -80 °C until used.

2.4. Dynamic aerosol toroid (DAT)

The stainless steel DAT used in this study (Fig. 1) measured 24 inches (60.9 cm) in diameter by 18 inches (45.7 cm) deep, thus providing a total volume of 133 liters. A variable speed motor (BHLW15L-120T-D2, Brother Gearmotors, Bridgewater, NJ, USA) rotated the DAT at 5 revolutions per minute (RPMs). The rotation of the DAT maintained the aerosolized particles in suspension and reduced the rate of physical loss through sedimentation [16, 37, 39]. Three ports, equally spaced on the circumference of the DAT and equipped with externally mounted hepa-filters (Fisher Scientific, 18-999-2574, Hampton, NH, USA), allowed for pressure equilibration during nebulization (introduction of air) and impingement (extraction of air).

For environmental control, the DAT was housed in a modified glove box designed to maintain temperatures from 2 °C to 40 °C (SS Series 600, 1695-03-36231, Terra Universal, Inc., Anaheim, CA, USA). Prior to aerosolization of PRRSV, the desired temperature and relative humidity were stabilized inside the glove box and DAT. Higher relative humidity

was achieved within the glove box by generating steam (Steam Dragon®, Newall Manufacturing Co., Chicago, IL, USA) using filtered (Millipore, Super-Q, ZFSQ115P4, cartridges, CDMB01204, CDAC01204, CP2001003, PMEG09002), sterilized water. Lower relative humidity was achieved by drying the air within the glove box (NitroWatch®, Terra Universal, 9670-00) with 99.995% pure N₂ gas (Chemistry stores, 1600.0085, Ames, IA, USA). Relative humidity and temperature within the glove box were continuously monitored (Dwyer Model 657 relative humidity/temperature transmitter, Dwyer Instruments, Inc., Michigan City, IN, USA). In addition, relative humidity and temperature within the DAT were independently measured using a probe (Vaisala, HMP46, Helsinki, Finland) connected to a temperature and humidity indicator (Vaisala, HMI41) inserted into the DAT.

2.5. Aerosolization of PRRSV

Suspension fluid consisting of 10 mL of stock PRRSV ($1 \times 10^{6.33}$ TCID₅₀/ mL), 40 mL sterile PBS (1X) (Invitrogen, 10010-064, Carlsbad, CA, USA), 0.1% (v/v) Rhodamine B dye (Sigma Chemical Co., R6626), and 0.01% (v/v) Antifoam A Emulsion (Sigma Chemical Co., A5758) was aerosolized into the DAT using a 24-jet Collison nebulizer (BGI Inc., CN60, Waltham, MA, USA). Previous research showed that Antifoam A Emulsion was non-virucidal for PRRSV [19]. The nebulizer was operated for 3 min on compressed air (Sears Roebuck, 00916734000, Hoffman Estates, IL, USA) at 40 P.S.I. producing: 80 L of free air per minute, a liquid generation rate of 66 mL/h, and a particle size of 1.9 µm [30]. The cloud was allowed to stabilize for 1 minute before sampling.

2.6. Impingers

SKC BioSampler® (SCK Inc., 225-9595, Eighty Four, PA, USA) impingers were used to collect samples from the DAT. Each impinger contained 20 mL of sterile PBS (1X) collection fluid and was operated for 1 minute. Impingers were operated at a vacuum pressure of $\leq (-0.05)$ ATM to ensure a constant flow rate of 12.5 L per minute (L per min). Flow rate was verified using a flow meter (Dwyer Instruments Inc., DW-806). Vacuum pressure was maintained using oil-less pumps (Fisher Scientific, S413801) and was monitored using a vacuum pressure gauge (Cato Western Inc., G-S4LM20-VAC-100, Tucson, AZ, USA).

2.7. Sampling of aerosolized PRRSV

A total of six samples were taken over the course of each replicate. The first sample was collected one minute post-nebulization. Thereafter, depending on the temperature, samples were collected at intervals appropriate to monitor the loss of infectivity over time. Since aerosolized infectious virus was more stable at lower temperature, the time interval between samples was greater at cooler temperatures than at warmer temperatures. Impinger samples were aliquoted into three 5 mL portions. Individual aliquots were used for: (1) microinfectivity assay (TCID₅₀) to determine the titer of infectious PRRSV; (2) quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) to determine total PRRSV RNA; (3) fluorometric analysis to determine the concentration of Rhodamine B. Microinfectivity and fluorometric analyses were performed immediately. Samples to be assayed by RT-PCR were frozen at -80 °C, completely randomized, and then submitted for analysis as a single set of samples.

2.8. Microinfectivity assay (TCID₅₀)

PRRSV was titrated on 96-well plates (Corning®, 3596) containing confluent 24 h-old MARC-145 cells. Samples containing virus were serially 10-fold diluted (10^0 to 10^{-5}) in MEM. Growth medium was discarded and 8 wells were inoculated with 100 μ L of sample at each dilution. After incubating for 2 h, the inoculum was discarded and 200 μ L of maintenance medium was added to each well. Plates were incubated at 37 °C in a humidified 5% CO₂ incubator for 48 h. Following incubation, cells were fixed with aqueous 80% acetone solution and stained with a fluorescein isothiocyanate-conjugated monoclonal antibody specific for PRRSV (SDOW17, 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 using the Spearman-Kärber method [21] and expressed as tissue culture infection dose 50 (TCID₅₀) per mL.

2.9. Polymerase chain reaction

PRRSV RNA for real-time RT-PCR amplification was extracted from 0.14 mL of sample with a QIAamp viral RNA minikit (Qiagen Inc., 210210, Valencia, CA, USA) following the protocols recommended by the manufacturer. Real-time RT-PCR quantification was performed using an ABI Prism® 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). Primers specific for PRRSV ORF7 were synthesized by Integrated DNA Technologies (Coralville, IA, USA) and MGB probes were synthesized by Applied Biosystems. The thermal profile for amplification of PRRSV RNA was a reverse transcription at 50 °C for 30 min, followed by enzyme activation at 95 °C for 15 min, then 40 cycles of denaturation at 94 °C for 15 s and a combined annealing/extension step at 60 °C for

60 s. For each assay, a standard curve was generated using standards containing PRRSV at 10^1 to 10^6 TCID₅₀ per mL and positive and negative control samples were included for quality control of the test with the unknowns.

2.10. Tracer quantification

Rhodamine B was used to quantify the physical loss of the aerosolized cloud containing PRRSV in the DAT through sedimentation over time [14, 22, 37, 39]. Impinger samples were transferred into cuvettes (Fisher Scientific, 14-385-942) and quantified using a fluorometer (9200-000, Turner BioSystems Inc., Sunnyvale, CA, USA) equipped with a green optical kit (9200-042, Turner BioSystems Inc.). The result was expressed in terms of the concentration of Rhodamine B present in the sample in parts per billion (PPB).

2.11. Statistical analysis

Within the DAT, infectious virus was lost over time through biological decay and physical loss due to sedimentation of airborne particles [16]. To calculate the biological decay of aerosolized infectious virus, it was necessary to adjust for the physical loss of infectious virus due to sedimentation, i.e., biological decay = total decay – physical loss. Using the concentration of Rhodamine B as a measure of physical loss [14, 22, 37, 39], the adjusted PRRSV infectious titers for each sample were estimated as shown in Equation 1, where subscripts represent time zero (0) or time “t”.

$$\text{Equation 1. Adjusted infectious virus titer} = \frac{\text{TCID}_{50} \text{ time } t}{\left(\frac{\text{tracer concentration time } t}{\text{tracer concentration time } 0} \right)}$$

Thereafter, the adjusted infectious PRRSV titers (\log_{10}) for the six sampling points were plotted against time. Subsequently, the T1/2 of PRRSV for each of the 18 experimental runs was calculated using linear regression analysis, as described by Bryan et al. [5]. Using the calculated T1/2 from the experimental runs, a non-linear regression model was selected to predict T1/2 at various temperature and relative humidity combinations. The model allowed for exponential decay of T1/2 using equation 2.

$$\text{Equation 2. } y_i = \alpha e^{-(\delta * Temp_i + \gamma * RH_i)} + \varepsilon_i,$$

where y_i represented the T1/2 for observation i , α was the intercept, and δ and γ represented the effect of temperature and relative humidity, respectively. All modeling was done using commercially-available statistical software (S-Plus 6.2, Insightful Corp., Seattle, WA, USA). The relationship between physical loss of the virus cloud and total detectable viral RNA was evaluated by comparing quantitative RT-PCR estimates of PRRSV RNA to quantitative (fluorometer) estimates of Rhodamine B dye in samples collected over time. To compare RT-PCR estimates of PRRSV concentration in TCID₅₀ equivalents to fluorometer estimates of Rhodamine B concentration in PPB, estimates for each sampling point were converted to a percent of the concentration in the cloud at time zero. This is shown in Equation 3, where “value” was either Rhodamine B concentration in PPB or concentration of PRRSV RNA in TCID₅₀ equivalents and subscripts represent time zero (0) or sample time “t”.

$$\text{Equation 3. Percent time}(t) = \frac{\text{value time}t}{\text{value time}0} \times 100$$

The difference between percent time(t) Rhodamine B and percent time(t) PRRSV RNA was calculated by subtracting one from the other. The difference was plotted against time

(minutes) for all sampling points and the slope of the line was derived by linear regression analysis. The slope of the regression line was compared to a slope of zero using analysis of variance.

Results

The experimental results showed that aerosolized PRRSV was least stable ($T_{1/2} = 3.6$ min) at 41.0 °C and 73.0% relative humidity and most stable ($T_{1/2} = 192.7$ min) at 5.0 °C and 17.1% relative humidity (Tab. I). In general, longer $T_{1/2}$ was associated with lower environmental temperature (Fig. 2) and/or lower relative humidity (Fig. 3). A non-linear regression model revealed that the effects of temperature ($p < 0.001$) and relative humidity ($p = 0.003$) on the $T_{1/2}$ of airborne PRRSV were statistically significant, but the interaction between temperature and relative humidity was not ($p = 0.21$). Based on this model, an equation was derived with which the $T_{1/2}$ of aerosolized PRRSV can be predicted for any combination of environmental temperature and relative humidity (Equation 4).

$$\text{Equation 4. } T_{1/2} = 339.037e^{(-0.0839*Temp) + (-0.00754*RH)}$$

Estimates of PRRSV $T_{1/2}$ for a range of temperature and relative humidity combinations is reported in Table II.

No statistical difference was detected between the percent time(t) of Rhodamine B and the percent time(t) PRRSV RNA by linear regression analysis ($p = 0.99$) (Fig. 4). This provided evidence that, under the conditions of this experiment, the decline in total PRRSV RNA was due to sedimentation and physical loss of particles, rather than degradation of viral RNA.

Discussion

Airborne transmission of viruses is a function of (1) the rate at which airborne virus is generated by infected hosts or other sources, (2) the stability ($T_{1/2}$) of infectious virus in aerosols, and 3) the virus dose required to infect a susceptible host via aerosol exposure. A key component in the process of aerosol transmission, $T_{1/2}$ reflects the likelihood that airborne virus will remain infectious a sufficient length of time to reach a susceptible host. The $T_{1/2}$ of infectious viruses in aerosols is affected by environmental factors, especially relative humidity and temperature [9, 14, 22, 33, 38]. In general, airborne viruses are more stable at lower temperatures, but viruses are not uniformly affected by environmental factors. For example, viruses with lipoprotein envelopes tend to be more stable at lower relative humidity and non-enveloped viruses more stable at higher relative humidity [1, 2, 10, 12-14, 27, 36, 39].

Consistent with these general trends, this experiment showed that PRRSV was more stable at lower temperatures and/or lower relative humidity. However, temperature exerted a greater effect on $T_{1/2}$ than relative humidity. That is, the effect of different levels of relative humidity on $T_{1/2}$ at low temperatures was distinct and measurable, but at higher temperatures the effect of levels of relative humidity on PRRSV $T_{1/2}$ was obscured by the rapid loss of virus infectivity (Figs. 2 and 3). Based on the results of this experiment, an equation was derived to predict PRRSV $T_{1/2}$ for any combination of temperature and relative humidity:

$$T_{1/2} = 339.037e^{(-0.0839*Temp) + (-0.00754*RH)}$$

Temperature and relative humidity are highly variable among geographic locations [24] and by season [20]. Indeed, temperature and relative humidity often vary greatly throughout the day. Given that $T_{1/2}$ is highly dependent upon temperature and relative humidity, it should be expected that the likelihood of aerosol transmission of PRRSV will vary by geographic location, season, and even throughout the day. Depending on specific local conditions, i.e. herd sizes, herd density, and climate, it is possible that aerosol transmission could be a significant route of PRRSV transmission in some regions of the world and not in others. The use of quantitative RT-PCR made it possible to monitor the presence of aerosolized PRRSV viral RNA independent from infectivity assays. In contrast to infectious PRRSV, PRRSV RNA was stable under the conditions evaluated in this study. Thus, a comparison of viral RNA and Rhodamine B dye, a physical tracer, found no significant difference in the slopes of the lines. This indicated that the loss of PRRSV RNA over time was due to sedimentation from the viral cloud, not decay of viral RNA. These results suggested that quantitative RT-PCR could be used as an ancillary measurement of physical loss of virus in future aerosol studies. However, there are no estimates on degradation of viral RNA in aerosols and it would be premature to substitute quantitative PCR estimates for a physical tracer, or abandon the use of physical tracers, until such estimates are available. An equally important observation was that quantitative RT-PCR results are independent of the quantity of infectious virus. Thus, it is not possible to interpret RT-PCR results in the context of the potential for virus transmission. Therefore, quantitative RT-PCR assays are extremely useful for laboratory stability studies, but detection of viruses in field samples using RT-PCR should be interpreted judiciously in the context of the potential for virus transmission.

Overall, the PRRSV T1/2 estimates reported begin to provide a quantitative basis for evaluating the risk of airborne transmission of PRRSV. Environmental conditions not evaluated in this experiment that may affect T1/2 of airborne PRRSV include ultraviolet radiation, wind speed, airborne debris, particle size, and composition of droplet. In addition to estimates on airborne stability, estimates on the quantity of PRRSV aerosolized by pigs and the dose required to infect susceptible animals is required to fully understand the likelihood of aerosol transmission and the circumstances under which it occurs.

Acknowledgements

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Tables and Figures

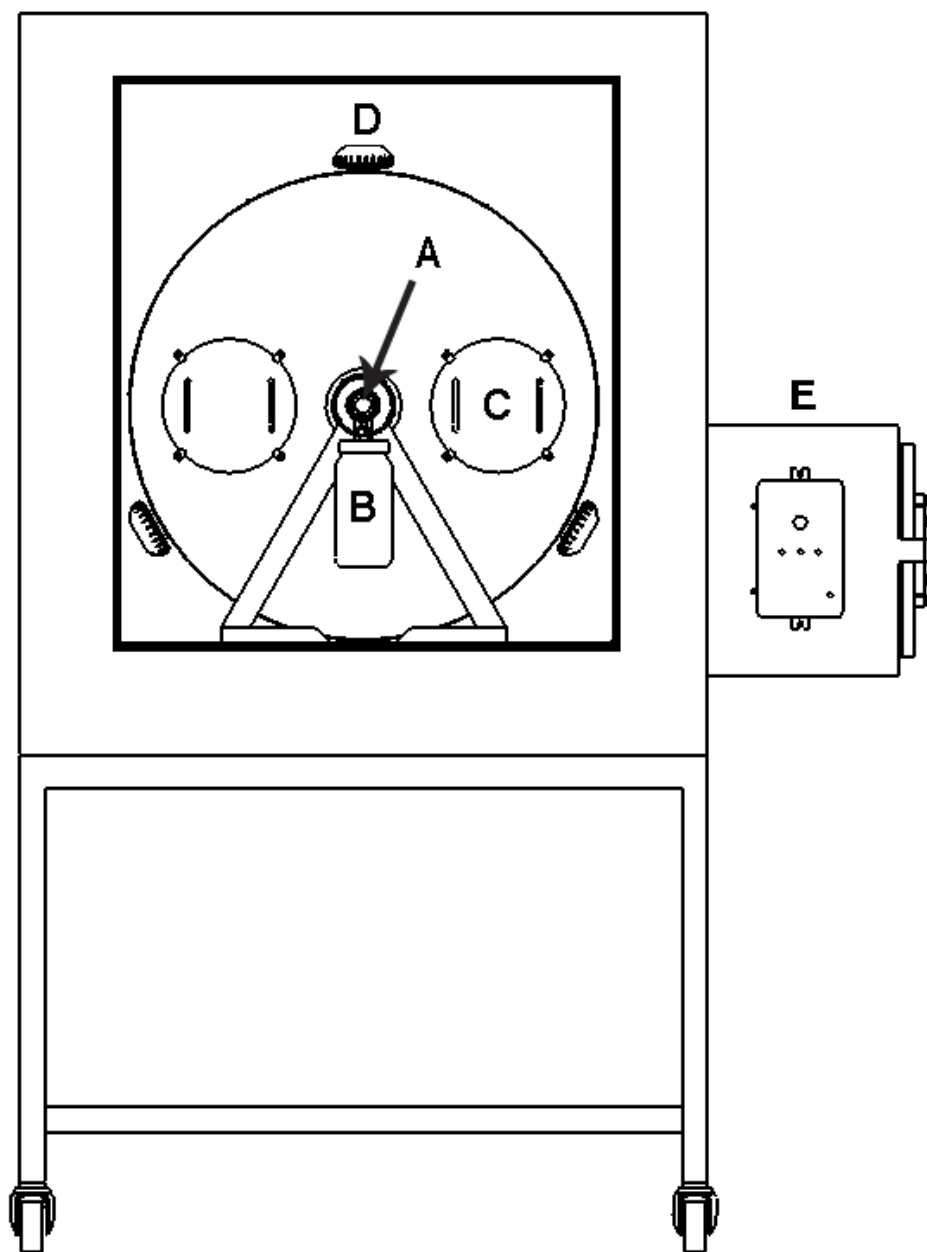


Figure 1. Dynamic aerosol toroid (DAT) housed in environmental glove box. (a) Port into environmental glove box for introducing and sampling aerosol cloud. (b) Placement of 24-jet Collison nebulizer for introducing aerosol or SKC BioSampler® impinger for sampling aerosol on outside of glove box. (c) Airtight access panel for decontamination of DAT. (d) Externally mounted hepa-filters (n=3) to filter makeup air during nebulization and impingement. (e) Airlock pass-thorough chamber and control panel for remote operation of DAT.

and relative

lf-life^b (min)

5.0	17.1	192.7
5.0	70.0	118.9
8.0	80.0	82.3
10.0	6.0	149.5
13.0	71.0	87.3
13.0	84.0	70.1
15.0	17.0	97.3
15.0	44.1	80.2
20.0	20.0	29.9
20.0	30.0	29.3
20.0	56.0	94.1
25.0	20.0	17.6
25.0	50.0	23.8
25.0	90.0	19.0
30.0	30.3	4.1
30.0	63.8	3.3
41.0	5.0	5.6
41.0	73.0	3.6

^aTemperature and relative humidity was measured using a probe connected to a temperature and humidity indicator.

^bObserved half-life was calculated using linear regression analysis of TCID₅₀ data collected from dynamic aerosol torrid.

Table II. Predicted half-lives of aerosolized infectious PRRSV by temperature and relative humidity combinations (min).

Temperature (°C)	Relative Humidity (%)								
	10	20	30	40	50	60	70	80	90
5	214.6 ^a	206.7	177.8	164.8	152.9	141.8	131.5	121.9	113.1
10	135.9	126.0	116.9	108.4	100.5	93.2	86.4	80.2	74.3
15	89.3	82.8	76.8	71.2	66.0	61.2	56.8	52.7	48.9
20	58.7	54.5	50.5	46.8	43.4	40.3	37.3	34.6	32.1
25	38.6	35.8	33.2	30.8	28.5	26.5	24.6	22.8	21.1
30	25.4	23.5	21.8	20.2	18.8	17.4	16.1	15.0	13.9
35	16.7	15.5	14.3	13.3	12.3	11.4	10.6	9.8	9.1
40	11.0	10.2	9.4	8.7	8.1	7.5	7.0	6.5	6.0

^aPredicted half-life was calculated using a non-linear regression model.

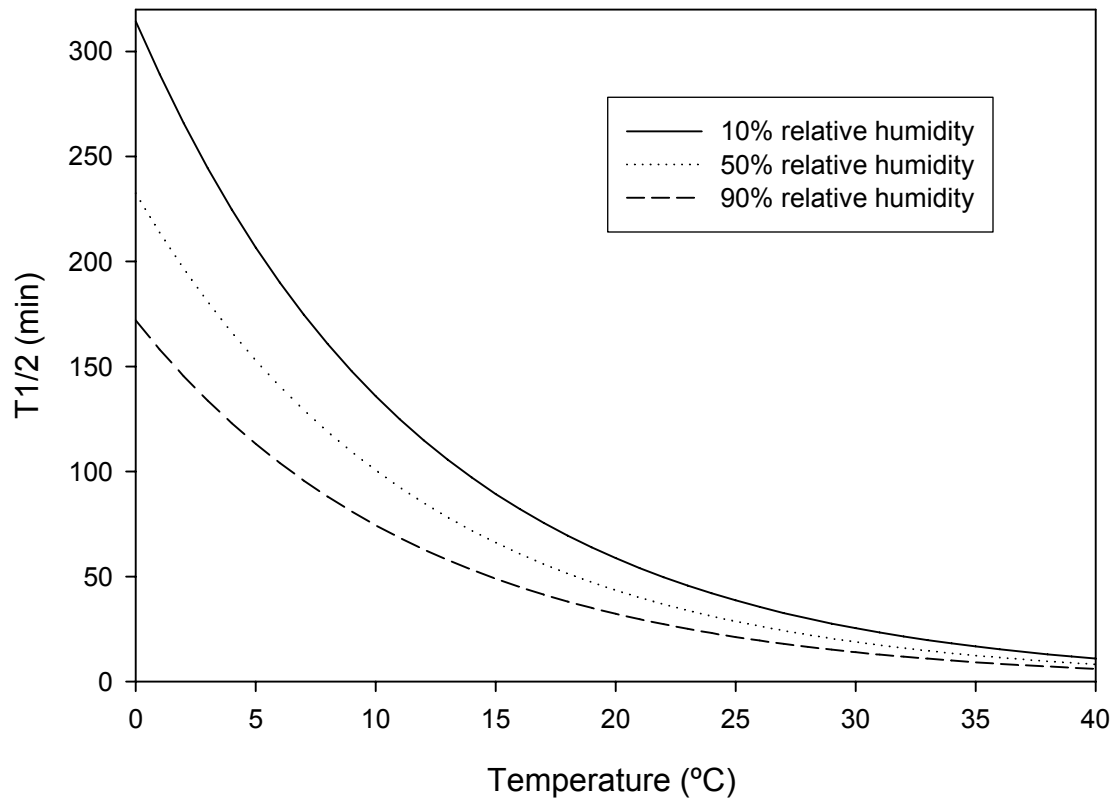


Figure 2. Predicted effect of temperature on half-life of aerosolized infectious PRRSV at 10%, 50%, and 90% relative humidity.

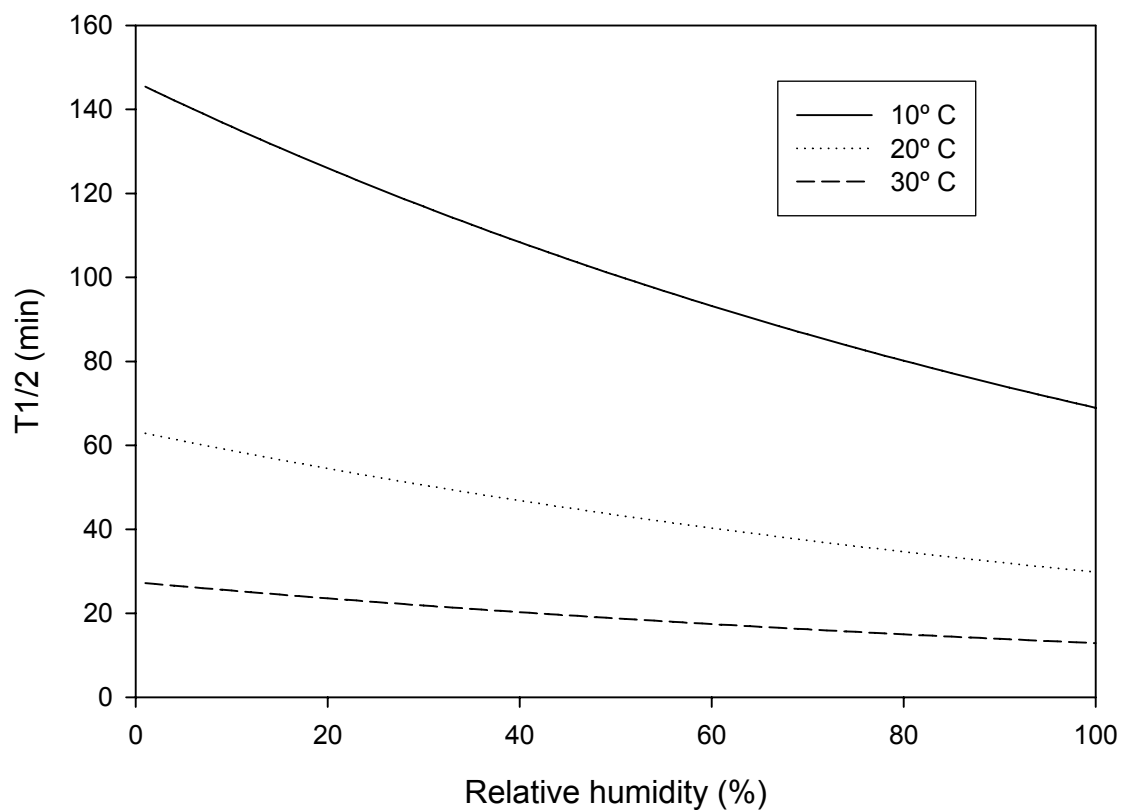


Figure 3. Predicted effect of relative humidity on half-life of aerosolized infectious PRRSV at 10°, 20°, and 30° C.

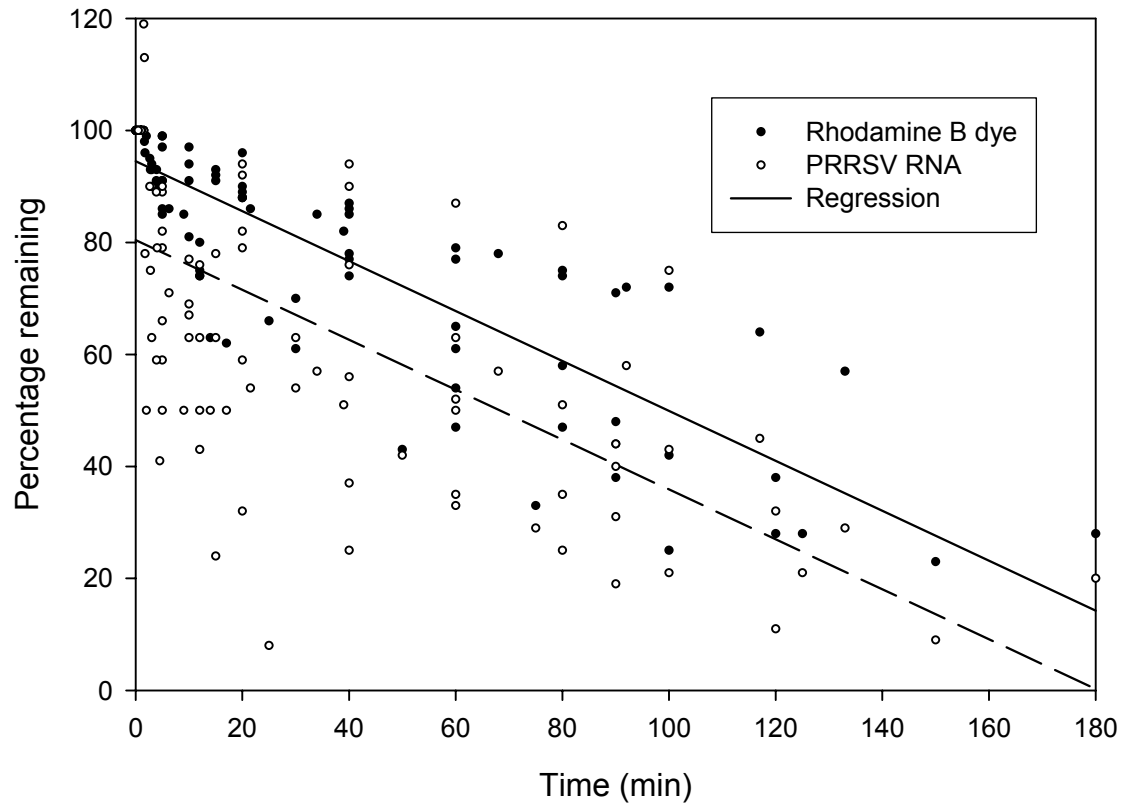


Figure 4. Percentage of Rhodamine B dye and PRRSV RNA remaining out of total aerosolized over time.

CHAPTER 6. A METHOD TO PROVIDE IMPROVED DOSE-RESPONSE ESTIMATES FOR AIRBORNE PATHOGENS IN ANIMALS: AN EXAMPLE USING PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

A paper submitted for publication to Veterinary Microbiology

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Abstract

This paper describes and validates a method that provides improved estimates of the probability that exposure to a specific dose of an airborne infectious pathogen will result in infection in animals. The method can be adapted to a variety of animal species and infectious pathogens. If replicated over a range of doses, the results can be used to derive a dose-response curve for the animal host and infectious pathogen of interest. This information is useful in estimating the likelihood of infection associated with exposure to airborne infectious microorganisms. Applications include predicting the risk of transmission associated with exposure to airborne pathogens, modeling the transmission of airborne pathogens, and determining requirements for effective exposure doses for vaccines delivered in aerosols.

Introduction

A wide variety of fungal, bacterial, and viral pathogens are transmissible in air (Tang et al., 2006). For humans, this includes re-emerging pathogens, e.g., *Mycobacterium tuberculosis* (Escombe et al., 2007), newly recognized pathogens, e.g., severe acute respiratory syndrome (SARS) coronavirus (Booth et al., 2005), and pathogens considered to have potential use as bioterrorism weapons (Atlas, 1998). For animals, this includes important zoonotic pathogens, e.g., avian influenza (Wong et al. 2006), and economically significant pathogens of livestock, e.g., foot and mouth disease (Alexandersen et al. 2002).

A comprehensive understanding of airborne spread relies upon quantitative estimates of the steps in the transmission process: aerosolization of the pathogen (source, quantity, and duration); dissemination of the airborne pathogen (droplet size and air movement); retention of infectivity over time (relative humidity, temperature, ultraviolet radiation); and probability of infection as a function of exposure dose. Although an important route of disease transmission, our understanding of the process of airborne transmission is rudimentary for most infectious agents. In large part, this is due to technical challenges in achieving quantitative estimates of excretion, dissemination, stability, and probability of infection by exposure dose.

This paper describes a method that provides improved estimates of the probability of infection as a function of exposure to an airborne infectious pathogen. To validate the method, the probability of infection by exposure dose to airborne porcine reproductive and respiratory syndrome virus (PRRSV) was determined for pigs. PRRSV is an enveloped RNA virus in the family *Arteriviridae* (Cavanagh, 1997) with a recognized potential for airborne transmission.

Materials and Methods

Experimental Design. The objective of this approach is to achieve precise dose-response estimates for aerosolized pathogens. Individual animals are exposed to a specific dose by breathing a known volume of air containing a known concentration of infectious pathogen. Following exposure, animals are individually housed and monitored for evidence of infection. The detection of specific antibodies or the pathogen in diagnostic specimens is evidence that the exposure dose resulted in infection. If replicated over a range of doses, the results can be used to derive a dose-response curve for the animal host and the pathogen of interest. This procedure can be adapted to a variety of animal species and airborne pathogens.

To validate the method, 7 replicates of 10 pigs per replicate were conducted. Within each replicate, 9 pigs were exposed to specific doses of airborne PRRSV. One pig served as an environmental sentinel, i.e., was in the room throughout the time treatments were administered, but was not intentionally exposed to PRRSV. Following treatment, pigs were housed individually to preclude transmission among animals. Serum samples collected at 0, 5, and 10 days post inoculation were assayed for the presence of PRRSV by virus isolation (VI) and reverse transcription-polymerase chain reaction (RT-PCR) to determine whether the exposure resulted in infection. The proportion of pigs infected at each exposure dose was used to derive the dose-response curve.

Source of airborne pathogen. A source of stable and quantifiable airborne infectious material is required for precise dose-response estimates. This condition may be

met by aerosolizing the pathogen into a rotating dynamic aerosol toroid (DAT) (Goldberg et al., 1958; Songer et al., 1967; Sattar et al., 1987). Rotation of the DAT, typically 3 to 5 revolutions per minute (RPM), maximizes the time the aerosolized particles remain in suspension (Goldberg et al., 1958; Songer et al., 1967; Sattar et al., 1984). Pathogen infectivity can be preserved by maintaining the DAT at a temperature appropriate for pathogen stability.

Physical loss of airborne pathogen within the DAT over time must be monitored in order to differentiate inactivation of infectious pathogen from sedimentation of particles. Depending on the pathogen, this can be done by testing samples collected at uniform intervals using quantitative polymerase chain reaction (PCR). Alternatively, an inert dye, e.g., Rhodamine B, can be incorporated into the suspension fluid containing the pathogen at the time of aerosolization. The decline in the concentration of pathogen or dye in the air within the DAT reflects physical sedimentation (Hermann et al., 2007).

For the PRRSV validation study, a stainless steel DAT (Figure 1) was constructed measuring 36 inches (91.4 cm) in diameter by 24 inches (60.9 cm) in depth (total volume of 400 liters). The DAT was rotated at 4 RPM using a variable speed motor (BHLW15L-120T-D2, Brother il Gearmotors, Bridgewater, NJ) and housed within a custom-built refrigeration unit with the capacity to maintain temperatures between 4°C to -20°C (Carroll Coolers, Inc., 20590, Carroll, IA) (Figure 1). The DAT was equilibrated at -19°C, a temperature at which infectious PRRSV is highly stable (Hermann et al., 2007), prior to aerosolization of the virus. To allow for pressure equilibration during introduction of air (nebulization) and extraction of air (impingement and pig exposure), three hepa-filters (Fisher Scientific, 18-999-2574,

Hampton, NH) were fixed to ports on the circumference of the DAT. Between replicates, the entire system was disassembled for cleaning and disinfection.

For the PRRSV validation study, isolate ATCC VR-2332 (American Type Culture Collection, Manassas, VA, USA; GenBank accession number PRU87392; Nelsen et al., 1999) was propagated in MARC-145 cells, as described in Hermann et al. (2007). To prepare one lot of high-titered PRRSV sufficient to conduct all the replicates, the propagated virus was pooled and concentrated (Cole-Parmer Instrument Co., 7518-00, Vernon Hills, IL, USA) such that approximately 2 liters of supernatant was reduced to approximately 200 ml of virus stock. The pooled virus stock was aliquoted into 10 ml volumes and stored at -80°C.

For aerosolization, a suspension fluid was prepared: 90 ml of PBS ((1X) (Invitrogen, 10010-064, Carlsbad, CA, USA), 10 ml of PRRSV stock solution ($1 \times 10^{6.3}$ TCID₅₀/ml), 0.1% (v/v) Rhodamine B dye (Sigma Chemical Co., R6626, St. Louis, MO), and 0.01% (v/v) Antifoam A Emulsion (Sigma Chemical Co., A5758, St. Louis, MO). The suspension fluid was aerosolized into the DAT using two 24-jet Collison nebulizers (BGI Inc., CN60, Waltham, MA) operating simultaneously for ten minutes on compressed air (Sears Roebuck, 00916734000, Hoffman Estates, IL) at 40 P.S.I. These parameters produced 80 liters of free air per minute, a liquid generation rate of 1.1 ml per minute, and a particle size of 1.9 µm (May, 1973) (Figure 1, Step 1).

Quantitation of airborne pathogen. Accurate estimates of infectious airborne pathogen per unit of air within the DAT are required to determine the exposure dose. To quantify pathogen concentration within the DAT, a measured volume of air within the DAT is sampled using an air sampling device and sampling protocol appropriate for the target pathogen. The specimen is then assayed for infectious pathogen using a quantitative

infectivity assay. The result is expressed in terms of quantity of infectious pathogen per volume of air within the DAT.

For the PRRSV validation study, SKC BioSampler® (SKC Inc., 225-9595, Eighty Four, PA) impingers were used to collect air samples from the DAT (Figure 1, Step 2). SKC BioSampler® impingers were selected on the basis of comparisons of PRRSV collection performance among various samplers (Hermann et al., 2006). Each impinger contained 20 ml of sterile PBS (1X) collection fluid and was operated for 2 minutes. Impingers were operated to ensure a constant flow rate of 12.5 liters per minute (L per min). Flow rate was verified using a flow meter (Dwyer Instruments Inc., DW-806, Michigan City, IN). Vacuum pressure was maintained using oil-less pumps (Fisher Scientific, S413801, Hampton, NH) and was monitored using a vacuum pressure gauge (Cato Western Inc., G-S4LM20-VAC-100, Tucson, AZ).

After nebulization, the aerosolized particles within the DAT were allowed to stabilize for 5 min before baseline air samples were taken. Thereafter, samples were collected immediately before and immediately after exposure of each pig to air within the DAT. After sampling, the collection fluid was assayed by: 1) PRRSV microinfectivity assay (TCID₅₀) to determine the titer of infectious virus per ml as described in Hermann et al. (2007); 2) PRRSV quantitative (qRT-PCR) to determine total genomic copies per ml as described in Wasilk et al. (2004).

Animal exposure to pathogen. To accurately calculate exposure dose, the volume of air respired by the animal during the exposure period is measured and multiplied by the concentration of aerosolized pathogen per unit of air. This requirement is met by measuring the volume of air delivered to the animal during exposure via an air-tight mask delivery

system fitted with a spirometer and a one-way valve. The one-way valve controls airflow such that air is inhaled from the DAT and then exhaled into a hepa-filtered chamber, thereby preventing contamination of the room.

For the PRRSV validation study, pigs were individually removed from isolation units on day 0, bled, and anesthetized using a combination of Telazol (5 mg; Fort Dodge Animal Health, Fort Dodge, Iowa), xylazine (250mg), and ketamine (250 mg; Fort Dodge Animal Health, Fort Dodge, Iowa). The xylazine and ketamine were used to reconstitute the lyophilized Telazol. The combination was administered intramuscularly at a dose of one mL per 22.5 kg of body weight. For exposure to the virus, the entire anterior portion of the head, i.e., snout and mouth, of the anesthetized pig was fitted with a canine surgical mask (SurgiVet, 32393B1, Waukasha, WI) attached with tubing (Fisher Scientific, 295736, Hampton, NH) to a one-way valve (Instrumentation Industries, Inc., BE-117, Bethel Park, PA) inserted into the DAT containing aerosolized infectious PRRSV (Figure 1 Step 3 and Figure 2). The cumulative volume (liters) of air inhaled by the animal during the exposure period was measured using a pediatric spirometer (Boehringer, 8805, Norristown, PA). In total, each pig was exposed to 10 liters of virus-laden air from the DAT.

Impinger samples were collected immediately before and after each pig exposure to determine average titer of PRRSV per liter of air during the exposure period. The average concentration of infectious pathogen per liter of air (pre- and post-exposure) was determined and multiplied by the volume of air respired (10 liters). The virus dose administered to each pig was calculated using the following equation:

$$\text{Exposure dose} = \frac{\text{TCID}_{50} \text{ estimate}}{\text{liter of air}} \times \text{liters of air respired}$$

Collection of biological samples. To determine if the exposure dose was sufficient to produce infection, biological samples are collected and assayed for the pathogen or pathogen-specific antibodies. If a sufficient number of replicates are conducted across a wide range of exposure doses, a dose response curve can be derived for the animal host and pathogen of interest.

For the PRRSV validation study, blood samples were collected from all pigs on 0, 5, and 10 DPE. Samples were collected using a single-use blood collection system (Vacutainer®, Becton Dickinson, Franklin Lakes, N.J.). Blood samples were centrifuged at 1000 x g for 10 min, after which the serum was harvested and stored at -80°C. At the end of each trial the samples were tested for evidence of PRRSV infection by VI (Hermann et al., 2005) and RT-PCR. Furthermore, to rule out previous exposure to PRRSV, day 0 serum samples were tested for PRRS virus-specific antibodies using a commercial enzyme-linked immunosorbent assay (ELISA) kit (HerdChek® PRRS Antibody 2XR Test Kit, IDEXX Laboratories, Westbrook, ME, USA).

Animals and animal care. Following exposure, each animal must be housed and handled to avoid inadvertent transmission among test animals by direct, indirect, and aerosol routes of exposure.

For the PRRSV validation study, animals were cared for in compliance with the requirements given in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (Federation of Animal Science Societies, 1999). Animal usage and procedures were approved by Iowa State University's Institutional Animal Care and Use Committee (IACUC #7-05-5931-S). To preclude transmission of pathogens, pigs approximately 5 weeks of age (16.85 kg) were individually housed in hepa-filtered (# P-007-

C-02-N2-IU-00-Z-2507B, Flanders Filters, Inc., Washington, NC, USA) isolation units (Barrier Systems, Inc., Toms River, NJ, USA). The isolation units were equipped with air, feed, and waste handling systems that maintained a biosecure environment and prevented transmission of PRRSV between pigs. Between replicates, the isolation units were cleaned and disinfected with chlorhexidine diacetate (Nolvasan® Solution; Fort Dodge Laboratories, Fort Dodge, Iowa) and then left empty for at least 48 hr with the heating system set at 32°C and the hepa-filter air system in operation.

Statistical analyses. In general terms, a dose-response model describes the probability of infection as a function of dose from exposure to a specified pathogen in a specified population. Several models have been used for dose-response modeling, including log-logistic, log-normal, exponential, Beta-Poisson, and Weibull-Gamma (Holcomb et al., 1999). The initial selection of a group of mathematical models to fit to the data depends on the type of outcome being measured (infection, clinical signs, biological marker) and the experimental design used to generate the data. Many different models will usually fit a given data set (Holcomb et al. 1999). Data on dichotomous variables, such as infection status as in this example, are commonly presented as a fraction of individuals that exhibit the given condition at a given dose level. For such endpoints, probability density models, e.g., logistic, probit, and Weibull, whose predictions lie between zero and one for any possible dose, are appropriate (U.S. Environmental Protection Agency, 2000; Holcomb et al. 1999).

The PRRSV validation study was conducted as an incomplete randomized block design where exposure doses were randomly allotted to experimental units (pigs) and varied between replicates. Each pig was considered an experimental unit because animals were individually housed in HEPA-filtered units. Calculation of the exposure dose was

complicated by the fact that, for the latter exposures in each replicate, the quantity of virus collected by the impingers was detectable by qRT-PCR, but below the analytical sensitivity of the microinfectivity (TCID₅₀) assay. Therefore, linear regression analysis was used to calculate the exposure dose. Specifically, the regression line was established by plotting impinger samples with calculable infectious titers (TCID₅₀) per ml vs. corresponding qRT-PCR genomic copies per ml. Thereafter, the concentration of infectious PRRSV per unit of air estimated using the following equations:

$$\text{TCID}_{50}\text{estimate} = \left(\frac{\text{RT - PCR copies}}{\text{ml of collection fluid}} \times \text{regression equation} \right) \times 20 \text{ ml liters of collection fluid}$$

where the regression equation corresponded to: TCID₅₀ = 2.10 + 0.25 RT-PCR copies.

The outcome was defined as a binary variable, i.e., aerosol exposure to a specific dose of PRRSV either produced infection or did not. Exposure doses ranged from $1 \times 10^{4.0}$ to $1 \times 10^{5.2}$ copies per ml, i.e., $1 \times 10^{2.7}$ to $1 \times 10^{3.5}$ TCID₅₀ per ml. Infection was determined based on detection of replicating virus in serum and/or detection of PRRSV specific antibodies. The probability of infection after aerosol exposure was modeled on the proportion of pigs that became infected by dose. For the dose response analysis, exposure doses were grouped in increments of $1 \times 10^{0.2}$ copies per ml.

For this application, both logit (log-logistic) and probit (log-normal) models were considered plausible models. The logit model was represented as $\ln \frac{P}{1-P}$, where P represents the probability that an individual chosen at random will become infected for a given dose “x”. P may be obtained from the logistic function

$$P = [1 + \exp(-\alpha - \beta x)]^{-1}, -\infty < x < \infty. \text{ Logit } P \text{ was estimated from the linear function logit } P$$

$= \alpha + \beta x$ (Govindarajulu, 2001). In the probit model, P was obtained from the distribution function of the normal distribution (Φ), where $P = (2\pi)^{-1/2} \int_{-\infty}^{\alpha+\beta x} \exp(-u^2/2) du, -\infty < x < \infty$.

Subsequently, probit P was estimated from the function probit $P = \text{normal deviate} = \alpha + \beta x$ (Govindarajulu, 2001). All modeling was done using commercially-available statistical software (S-Plus 6.2, Insightful Corp. Seattle, WA).

Results

Prior to exposure, PRRS virus infection-free status was determined on the basis of serum samples collected from individual pigs on -7 and 0 DPE and assayed for PRRS virus and anti-PRRS virus antibodies. All serum ELISA S/P ratios were negative on -7 and 0 DPE, indicating that the animals had not had prior exposure to PRRSV. Likewise, all serum samples collected from pigs were VI negative and RT-PCR negative on DPE 0.

Following exposure, PRRS virus infection status was determined on the basis of VI and RT-PCR results on serum samples collected from individual pigs on 5 and 10 DPE. Animals were considered infected if DPE 5 and 10 serum samples were positive by both VI and RT-PCR. All samples from negative control animals tested negative by VI and RT-PCR. The numbers of pigs infected by number of pigs exposed to aerosolized PRRSV by dose are presented in Table 1. These data were subsequently analyzed and the estimated dose response curve calculated (Figure 2).

Discussion

The objective of this paper was to describe an improved method for calculating the probability that a defined exposure dose results in the transmission of a specific airborne pathogens in animals. Historically, most transmission experiments in aerobiology have focused on testing whether the pathogen of interest could be transmitted via aerosol, i.e., sentinel animals became infected by exposure or not. A variety of experimental designs have been used to that end, but most have failed to provide for an estimate of the exposure dose. In addition, this approach usually fails to take into account a variety of factors that affects exposure doses, e.g., differences in excretion rates of infected donor animals (Alexandersen et al., 2003), respiratory tidal volumes of the host animal (Alexandersen et al. 2002), sedimentation and/or dilution rates of airborne particles (Morawska, 2006), rate of airborne pathogen inactivation (Hermann et al., 2007), and air sampling methods which produce inconsistent estimates of pathogen concentration (Bourgueil et al., 1992). Consequently, descriptive transmission experiments are difficult to interpret or apply to the field.

The method described applies several solutions to the problem of arriving at a precise estimate of exposure dose. First, a stable and quantifiable source of aerosolized pathogen is achieved by nebulizing pathogen into a rotating dynamic aerosol toroid (DAT) housed under controlled environmental conditions. The DAT physically reduces the rate of pathogen-contaminated droplet loss through sedimentation (Goldberg et al., 1958; Songer et al., 1967; Sattar et al., 1984). In addition, the air in the DAT can be conditioned to stabilize pathogen infectivity. For example, in the PRRSV validation study, virus infectivity was preserved by holding the DAT at -19°C (Hermann et al., 2007). Virus quantification is achieved by sequential air sampling and testing of samples in quantitative pathogen-specific assay. Thus,

in the PRRSV study, the concentration of virus in the DAT was monitored using a validated air sampling procedure in conjunction with quantitative virus assays (Hermann et al., 2006). An further benefit of the repeated measures design is that estimates of virus concentration can be calculated using linear regression analysis (qRT-PCR genomic copies per ml vs TCID₅₀ per ml) when the concentration of virus is below the threshold of detection. Second, the volume of air respired by the animal during the exposure period may be accurately measured using a pediatric spirometer connected to the DAT via a one way valve. The one way valve functions to direct pathogen-laden air from the DAT to the animal (or the impinger) during inspiration and then into a containment vessel at exhalation.

Delivering a known volume of air containing a known concentration of pathogen results in precise estimates of exposure dose. The resultant dose response curves are based on robust estimates of exposure doses derived from quantitative measures (PCR and microtitration) of the concentration of airborne pathogen per liter of air and the exact volume of air respired by the host animal during exposure.

Although generally poorly described, aerosol transmission of infectious airborne pathogens is often considered an important route of transmission and a significant challenge to disease prevention and control (Gillespie et al., 2000, Wilkinson et al., 1977). In particular, the need for a quantitative approach to describe airborne contagion based on the relationship between exposure dose and infection has been a recognized need (Donaldson et al., 1987; French et al., 2002; Gillespie et al., 2000; Phillpotts et al. 1997). This method can be used to fit this requirement for a variety of airborne pathogens and animal hosts.

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Tables and Figures

Table 1. Number of pigs infected with PRRSV as a function of exposure dose.

Total exposure dose (qRT-PCR ^a)	Total exposure dose (TCID ₅₀)	Infected/Exposed ^b
$<10^{4.0}$	$<10^{3.1}$	2/4
$10^{4.2}$	$10^{3.2}$	2/3
$10^{4.4}$	$10^{3.2}$	8/11
$10^{4.6}$	$10^{3.3}$	10/14
$10^{4.8}$	$10^{3.3}$	14/15
$10^{5.0}$	$10^{3.4}$	12/13
$10^{5.2}$	$10^{3.5}$	3/3

^aGenomic copies of PRRSV

^bNumber of animals infected / Number of animals exposed

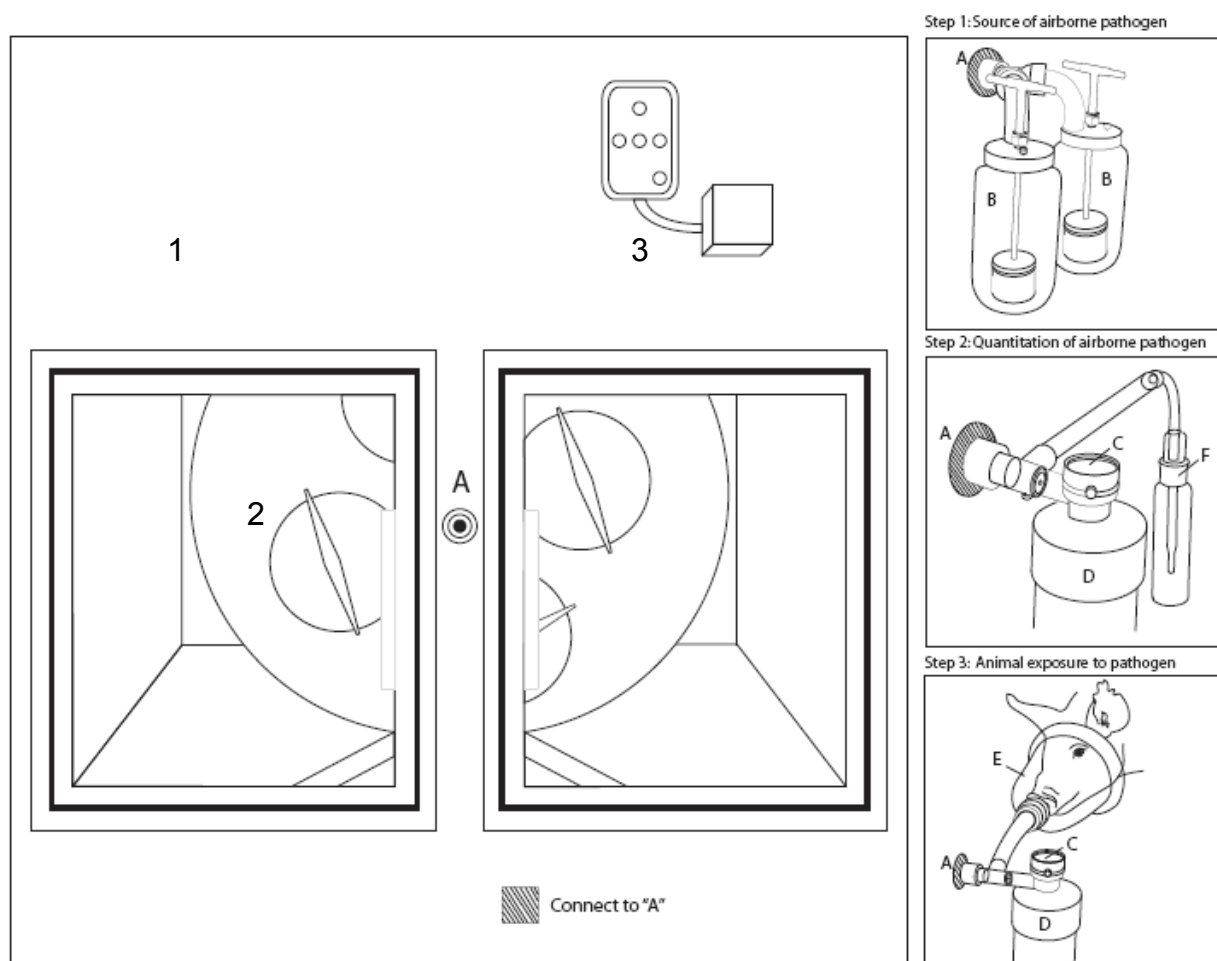


Figure 1. Diagram of methodology used to derive dose response curves for aerosolized pathogens. 1= Modified refrigerator-freezer unit, 2= Dynamic aerosol toroid (DAT), 3=External control for temperature and rotation of DAT. A= External connection for introduction of aerosol and extraction of air, B= Collision nebulizer, C= pediatric spirometer, D= hepa-filtered chamber, E= Canine mask, F= Air impinger.

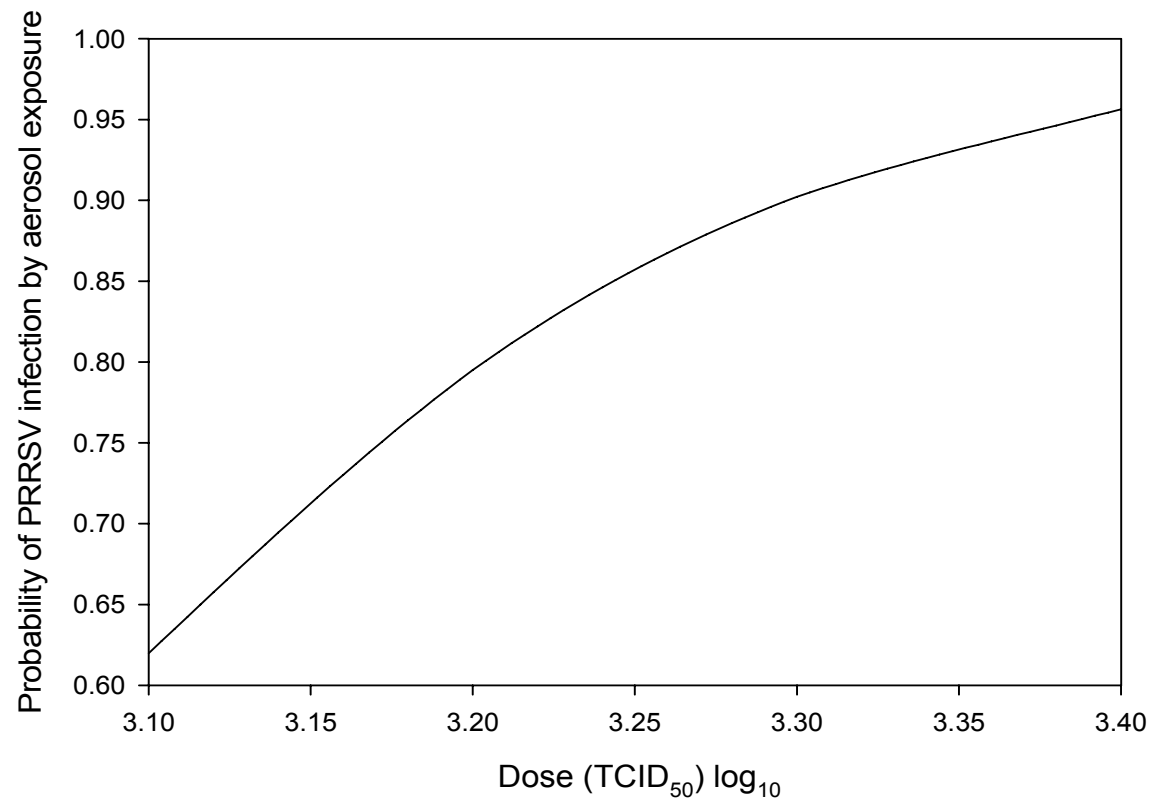


Figure 2. Dose response curve for aerosol route of exposure

CHAPTER 7. GENERAL CONCLUSIONS

Although considered an important route of transmission, the role airborne PRRSV plays in disease spread remain unanswered. To ascertain answer to these questions, five quantitative studies on the factors influencing the likelihood of transmission: 1) virus excretion, 2) stability of aerosolized virus, and 3) infectious dose required were conducted.

The first study described sampling parameters for increased recovery and detection of airborne PRRSV. The results demonstrated that collection medium, impinger model, and sampling time, independently influenced the recovery and detection of PRRSV. Based on the results of this experiment, the air sampling parameters were optimized for sampling i.e. recovery/detection of PRRSV and implemented throughout the remaining experiments.

The second study estimated the analytical sensitivity of four air samplers for PRRSV. Based on the following results no difference in analytical sensitivity between samplers for collection of artificially produced aerosols containing cell culture propagated PRRSV exists. Detection thresholds obtained from this experiment were used for reference in pig excretion studies.

The third study attempted to characterize the excretion of PRRSV in respirations from acutely infected pigs. Based on the following results acutely infected pigs did not aerosolize PRRSV or the quantity of virus excreted was below the analytical sensitivity (detection threshold) of current sampling and/or assay systems at the individual pig level.

The forth study described the stability of airborne infectious porcine reproductive and respiratory syndrome virus (PRRSV) as a function of temperature and relative humidity. Analysis of the results showed that aerosolized PRRSV was more stable at lower

temperatures and/or lower relative humidity, but temperature had a greater effect on the T1/2 of PRRSV than relative humidity. Based on these results, an equation was derived to predict the T1/2 of infectious airborne PRRSV for any combination of environmental temperature and relative humidity.

The last study described and validated a method to improve dose response estimates for aerosolized PRRSV. The results demonstrated pigs were readily infected with airborne PRRSV. Based on these results a dose response curve was derived for PRRSV.

Overall the comprehensive results indicate that the probability of airborne transmission of PRRSV is dependent on a delicate sequence of events. Strategies to disrupt this cycle will reduce the risk of aerosol transmission. Further studies to quantify excretion of airborne PRRSV by groups of pigs are needed. Additionally, studies to describe the slope and lower plateau of dose response curves for PRRSV are essential. This additional information will be useful in prediction models to forecast the spread of PRRSV and assist in developing prevention strategies to control aerosol transmission.

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