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Short communication

A method to provide improved dose–response estimates for airborne pathogens in animals: An example using porcine reproductive and respiratory syndrome virus

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ABSTRACT

This paper describes a method to provide improved probability estimates that exposure to a specific dose of an airborne infectious pathogen will result in animal infection. Individual animals were exposed to a specific dose of airborne pathogen. Following exposure, animals were individually housed and monitored for evidence of infection. The detection of specific antibodies and/or the pathogen in diagnostic specimens was 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 a variety of animal species and infectious pathogens. 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.

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1. 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 and Yuen, 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.

The objective of this study was to provide a method to achieve precise dose–response estimates for airborne pathogens. Porcine reproductive and respiratory syndrome virus (PRRSV) an enveloped RNA virus in the family *Arteriviridae* (Cavanagh, 1997) with a recognized potential for airborne transmission was used to validate the method.

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2. Materials and methods

2.1. Experimental design

The study was conducted as an incomplete randomized block design where pigs were exposed to a dose of airborne PRRSV. Seven replicates composed 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.

2.2. Source of airborne pathogen

A stainless steel DAT (Fig. 1) was constructed measuring 36 in. (91.4 cm) in diameter \times 24 in. (60.9 cm) in depth (total volume of 400 l). 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 and -20°C (Carroll Coolers, Inc., 20590, Carroll, IA) (Fig. 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.

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 using a Prep/Scale spiral wound ultra-filtration module (Model CDUF 001 LH, Millipore, Billerica, MA, USA) such that approximately 2 l 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 ($1\times$) (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 10 min 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) (Fig. 1, Step 1).

2.3. Quantitation of airborne pathogen

SKC BioSampler[®] (SKC Inc., 225-9595, Eighty Four, PA) impingers were used to collect air samples from the DAT (Fig. 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 ($1\times$) collection fluid and was operated for 2 min. Impingers were operated to ensure a constant flow rate of 12.5 l per minute (l/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).

2.4. Animal exposure to pathogen

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 1 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 (Fig. 1 Step 3). 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 l 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 l). The virus dose

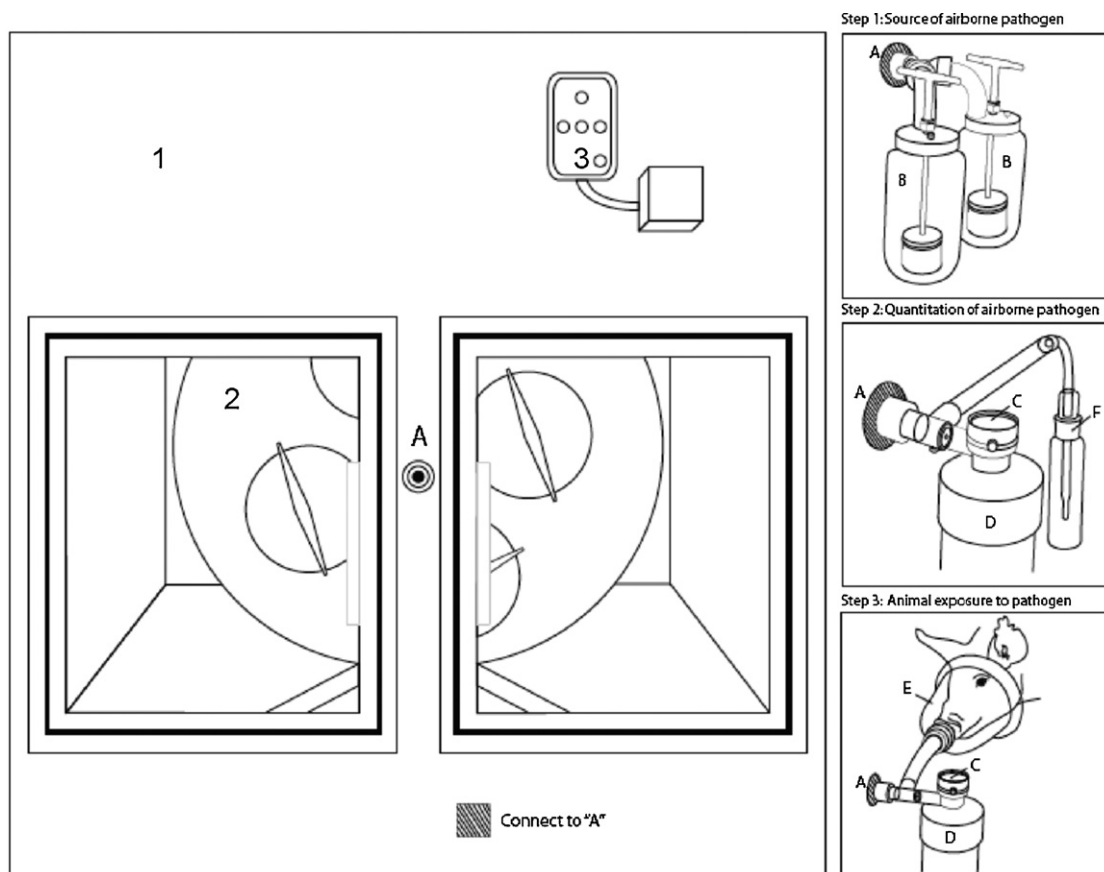


Fig. 1. Diagram of methodology used to derive dose–response curves for aerosolized pathogens. 1, Modified refrigerator–freezer unit; 2, Dynamic aerosol toroid (DAT); 3, External control for temperature and rotation of DAT. A, External connection for introduction of aerosol and extraction of air; B, Collision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

administered to each pig was calculated using the following equation:

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

2.5. Collection of biological samples

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, NJ). Blood samples were centrifuged at $1000 \times g$ 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).

2.6. Animals and animal care

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). Pigs were approximately 5 weeks of age ranging in weight from 11.4 to 22.7 kg with a mean weight of 16.85 kg. To avoid inadvertent transmission among test animals by direct, indirect, and aerosol routes of exposure, pigs 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) and handled separately. 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 h with the heating system set at 32°C and the hepa-filter air system in operation.

2.7. Statistical analyses

Each pig was considered an experimental unit because exposed animals were individually housed in HEPA-filtered units. The outcome was defined as a binary variable, i.e.,

aerosol exposure to a specific dose of PRRSV either produced infection or did not. The explanatory variable (dose) was continuous. The probability of infection after aerosol exposure was modeled on the proportion of pigs that became infected by dose. For this application, both logit (log-logistic) and probit (log-normal) models were considered plausible models. The logit model was represented as $\ln(P/1 - P)$, 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 $P = [1 + \exp(-\alpha - \beta x)]^{-1}$, $-\infty < x < \infty$ logit P was estimated from the linear function $\text{logit } P = \alpha + \beta x$ (Govindarajulu, 2001). In the probit model, P was obtained from the distribution function of the normal distribution (Φ), where

$$P = (2\pi)^{-1/2} \int_{-\infty}^{\alpha + \beta x} \exp(-u^2/2) du, \quad -\infty < x < \infty$$

Subsequently, probit P was estimated from the function $\text{probit } P = \text{normal deviate} = \alpha + \beta x$ (Govindarajulu, 2001). Parameters α and β and their standard deviations for each model were estimated using the method of maximum likelihood and fitted models were subsequently used to predict the dose and 90% confidence interval for a given probability of infection. The fit of the models was evaluated using an analysis of deviance, where a P -value < 0.05 was considered significant. All modeling was done using commercially available statistical software (S-Plus 6.2, Insightful Corp. Seattle, WA).

Quantitative RT-PCR data from serum samples were analyzed using fixed effect models with day as a repeated measure using MANOVA (JMP[®], SAS Institute Inc., Cary, NC, USA). The dependent variable was viremia. The independent variables were dose and time. The interaction

of dose \times time was examined for statistical significance. Data were reported as least squares means. A significance level of less than 0.05 was required as the minimum acceptable P -value.

3. 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 .

Exposure doses administered to pigs ranged from $1 \times 10^{4.0}$ to $1 \times 10^{5.2}$ genomic copies per ml, i.e., $1 \times 10^{2.7}$ to $1 \times 10^{3.5}$ TCID₅₀ per ml. The latter exposures in each replicate the quantity of virus and 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 where the regression equation corresponded to: $\text{TCID}_{50} = 2.10 + 0.25 \text{ RT-PCR copies}$. Thereafter, the concentration of infectious PRRSV per unit of air estimated using the following equation:

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

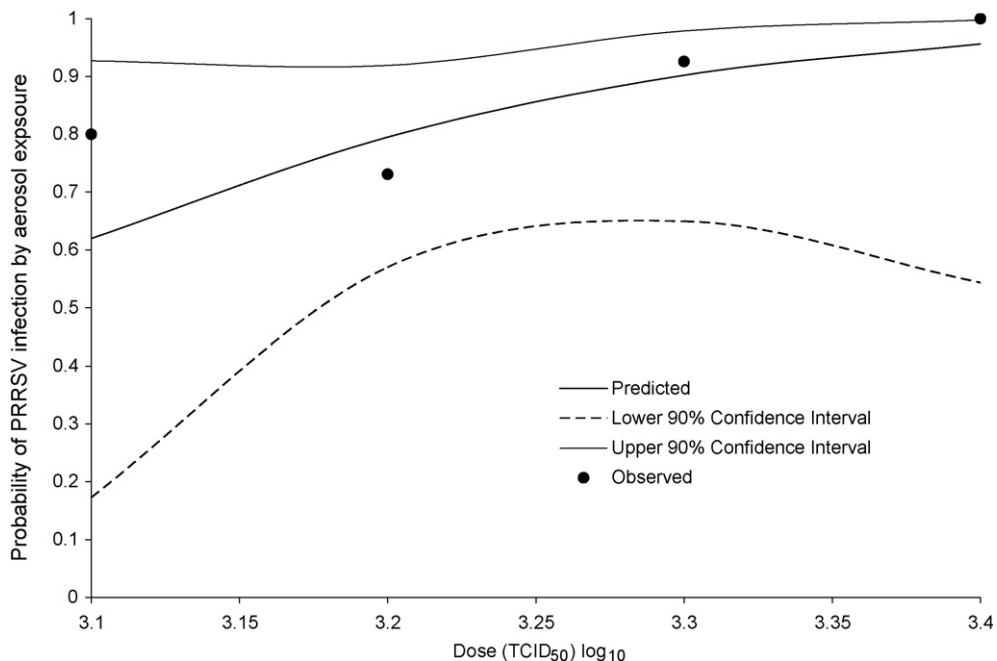


Fig. 2. Dose–response curve for aerosol route of exposure.

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 |

^a Genomic copies of PRRSV.

^b Number of animals infected/Number of animals exposed.

Table 2
Viremia of PRRSV infected pigs by day post inoculation

| Total exposure dose (qRT-PCR ^a) | Viremia ^a | | |
|---|----------------------|--------------------|--------------------|
| | Day 0 | Day 5 | Day 10 |
| <10 ^{4.0} | 10 ^{0.00} | 10 ^{6.14} | 10 ^{6.14} |
| 10 ^{4.2} | 10 ^{0.00} | 10 ^{6.35} | 10 ^{6.40} |
| 10 ^{4.4} | 10 ^{0.00} | 10 ^{3.64} | 10 ^{5.77} |
| 10 ^{4.6} | 10 ^{0.00} | 10 ^{4.67} | 10 ^{6.01} |
| 10 ^{4.8} | 10 ^{0.00} | 10 ^{4.48} | 10 ^{5.61} |
| 10 ^{5.0} | 10 ^{0.00} | 10 ^{4.44} | 10 ^{6.29} |
| 10 ^{5.2} | 10 ^{0.00} | 10 ^{5.84} | 10 ^{6.64} |

^a Genomic copies of PRRSV.

For the dose–response analysis, exposure doses were grouped in increments of $1 \times 10^{0.2}$ copies per ml (Fig. 2).

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 serum samples were positive on DPE 5 and 10 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. For pigs infected with PRRSV, no dose \times time interactions ($P > 0.40$) were observed for serum titers (Table 2).

4. Discussion

The specific role aerosolized pathogens play in airborne transmission of disease has not been well characterized (Douwes et al., 2003). 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 “animal in a box” 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 (Bourgeois et al., 1992). Consequently, descriptive transmission

experiments are difficult to interpret or apply to the field. Thus the objective of this paper was to describe an improved methodology for calculating the probability that a dose of airborne pathogen will result in animal infection. 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. 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 was achieved by nebulizing pathogen into a rotating dynamic aerosol toroid (DAT) housed under controlled environmental conditions. Rotation of the DAT, typically 3–5 revolutions per minute (rpm), reduces the rate of pathogen-contaminated droplet loss through sedimentation (Goldberg et al., 1958; Songer, 1967; Sattar et al., 1987). Although particle suspension is maximized, 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). Pathogen infectivity can be preserved by maintaining the DAT at a temperature appropriate for pathogen stability. For example, in this study PRRSV virus infectivity was preserved by holding the DAT at -19°C (Hermann et al., 2007).

Second, accurate estimates of infectious airborne pathogen per unit of air within the DAT were determined. Pathogen quantification is achieved by taking sequential air samples using an air sampling device and sampling protocol appropriate for the target pathogen. Collection medium from the air-sampling device is tested using a quantitative pathogen-specific assay. The result is expressed in terms of quantity of infectious pathogen per volume of air within the DAT. To quantify concentration of PRRSV within the DAT, air samples were taken using a validated sampling procedure (Hermann et al., 2006). The collection fluid was then assayed for infectious pathogen using a quantitative infectivity assay and real time RT-PCR. A further benefit of taking sequential samples 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 infectious virus is below the threshold of detection.

Third, the volume of air delivered to the animal during exposure was measured via an airtight mask delivery system fitted with a pediatric spirometer and 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 hepa-filtered chamber at exhalation, thereby preventing contamination of the room. Delivering a known volume of air containing a known concentration of pathogen results in precise estimates of exposure dose.

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. The resultant dose–response curves are based on robust estimates of exposure doses derived from quantitative measures 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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