A method to quantify infectious airborne pathogens at concentrations below the threshold of quantification by culture

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Abstract

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

Résumé

En aérobiologie, les études dose-réponse sont utilisées pour estimer le risque d'infection que représente pour un hôte susceptible l'exposition à une dose spécifique d'un agent pathogène en suspension dans l'air. Dans un environnement de recherche, les facteurs spécifiques à l'hôte et à l'agent qui affectent le continuum dose-réponse peuvent être tenus pour compte dans le design expérimental, mais l'obligation de déterminer précisément la dose d'agent pathogène à laquelle l'hôte a été exposée représente souvent un défi. Par définition, la quantification des agents pathogènes viables en suspension dans l'air est basée sur la culture des microorganismes, mais certains agents pathogènes aériens sont transmissibles à des concentrations inférieures au seuil de quantification par culture. Dans cet article nous présentons une approche pour le calcul de la dose d'exposition à des niveaux non-quantifiables microbiologiquement en utilisant une application du modèle de réaction en réservoir avec agitation continue (CSTR) et la validation de cette approche en utilisant le colorant rhodamine B comme substitut à des agents pathogènes microbiens mis en aérosol dans un tore dynamique (DAT).

(Traduit par Docteur Serge Messier)

Introduction

Airborne transmission poses a major challenge to the control of human and animal pathogens. For humans, airborne transport has been linked to the transmission of *Coccidioides immitis* (1); *Mycobacterium tuberculosis* (2); *Legionella* spp. (3); smallpox virus (4); and a variety of other pathogenic fungi, bacteria, and viruses (5–8). For animals, some of the most economically significant pathogens are transmitted in bioaerosols, such as foot-and-mouth disease virus (9), classical swine fever virus (10), and porcine respiratory and reproductive syndrome virus (11). Of importance to both human and animal health are major zoonotic pathogens transmitted via aerosols, including influenza virus (12,13), severe acute respiratory syndrome (SARS) coronavirus (14), *Yersinia pestis* (6), *Bacillus anthracis* (15), and others.

In aerobiology, dose-response curves are useful for describing the probability (y-axis) that a specific dose (x-axis) of an airborne pathogen will produce infection in a susceptible host (5,16). Under experimental conditions, dose-response curves can be derived by individually exposing susceptible animal hosts to a known quantity of pathogen and then monitoring each animal for evidence of infection under conditions that preclude the possibility of infection from all other sources (17,18). The proportion of individuals that become infected at each dose provides the raw data upon which the dose-response curve is based.

A variety of statistical techniques can be used to analyze the dose-response relationship, with ID_{50}, the dose required to infect 50% of the population, being the most useful summary statistic of the dose-response for any defined pathogen-host system (19,20). A standard dose-response curve is defined by 4 parameters: the baseline (bottom), the maximum response (top), the slope of the curve, and the mid-point of the curve. But the exact parameters of a dose-response curve depend on the pathogen (21), the strain or isolate of the pathogen (19), the host species (22), and specific host factors, such as age and immune status (23).

In a research setting, host- and pathogen-specific factors that affect the dose-response curve can be accounted for by careful experimental design. A larger challenge is the requirement to determine

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

Exchange (x)[a]	C_t/C_{in} [b]	Target retained in the CSTR (%)
1	$1 - e^{-1}$	37.0
2	$1 - e^{-2}$	14.0
3	$1 - e^{-3}$	5.0
4	$1 - e^{-4}$	1.8
5	$1 - e^{-5}$	0.7

[a] x = one complete exchange of the volume of the CSTR.

[b] Mass balance of target concentration.

C_t — target concentration at time = t; C_{in} — target input concentration time at time = 0.

the dose of infectious pathogen to which the host was exposed. Estimation of the exposure dose requires measurements of the total volume of air respired by the host and the concentration of viable airborne pathogen, for example, liters of air respired by the susceptible host × pathogen concentration per liter of air = exposure dose. In domestic animals, total respired air can be measured using spirometric instrumentation (18).

Estimates of the concentrations of infectious airborne pathogens are often more difficult to achieve. By definition, quantification of viable airborne pathogens is based on techniques that require the micro-organism to replicate in culture. Culture-based methods are less analytically sensitive than contemporary molecular techniques, e.g., polymerase chain reaction (PCR), but molecular assays are not a good substitute because they do not differentiate between infectious and non-infectious micro-organisms (5,19). If the pathogen is not highly transmissible, i.e., if transmission requires a large exposure dose, the dose-response curve may be determined despite the requirement to quantify infectious micro-organisms in culture. Commonly, airborne pathogens are transmissible at concentrations below the threshold of quantification by culture. Under these circumstances, the exposure dose, and hence the probability of transmission, is incalculable (16,24). In this paper, we present an engineering approach to calculate the exposure dose at microbiologically unquantifiable levels.

The continuous-stirred tank reactor (CSTR) is a vessel characterized by steady-state and uniform internal conditions due to mixing. The reactions and processes occurring within the CSTR may be defined for the conditions of the vessel. For example, as shown in Table I, the concentration of a target within a CSTR can be predicted at any time (t) using the equation:

$$C_t = C_{in}\,(1 - e^{-t(Q/V)}) = C = C_{in}\,(1 - e^{-x})$$

where:

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

Continuous-stirred tank reactors are widely used in a variety of industrial, chemical, and biological applications, including bioreactors, fermentation vessels, and wastewater treatment. Goldberg et al (25) introduced the use of a continuously rotating drum (dynamic aerosol toroid) to experimental aerobiology as a method to maintain and study infectious particles suspended in aerosols over time. A rotating dynamic aerosol toroid (DAT) housed in an environment that preserves the pathogen's infectivity, e.g., held at temperatures below freezing, is a CSTR. As such, the concentrations of the airborne components within the DAT can be predicted at time *(t)* if the exchange volumes are known. This paper provides experimental evidence to support this concept and discusses the application of this approach to the problem of estimating the concentration of airborne pathogens at microbiologically unquantifiable levels.

Materials and methods

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

A stainless steel 400 liter DAT was constructed based on the description provided by Goldberg et al (25). For temperature control, the DAT was housed in a custom-built refrigeration unit (Carroll Coolers Inc., Carroll, Iowa, USA) maintained at −4°C. During operation, the DAT rotated at 4 RPM (Brother International Gearmotors, Bridgewater, New Jersey, USA). Three HEPA-filters (Fisher Scientific, Hampton, New Hampshire, USA) were fixed to ports on the periphery of the DAT to allow for pressure equilibration during nebulization and impingement. The entire system was disassembled and cleaned between each of the 10 replicates.

In each replicate, approximately 50 mL of a 1× phosphate buffered saline (PBS; Thermo Scientific, Rockford, Illinois, USA) solution containing 0.08% v/v; rhodamine B (Sigma Chemical Company, St. Louis, Missouri, USA) and 0.1% v/v Antifoam A Emulsion (Sigma Chemical Company) was nebulized into the DAT using a 24-jet Collison nebulizer (BGI, Waltham, Massachusetts, USA) operating at 40 PSI. According to the manufacturer's specifications, these parameters aerosolized the solution at a rate of 1.1 mL per min and produced particles 1.9 μm in diameter. After nebulization and prior to sampling, the cloud was allowed to equilibrate within the DAT for 60 min. This allowed for complete mixing, sedimentation, and thermal equilibration of aerosolized rhodamine B.

Air samples were collected using sterile AGI-30 glass impingers (Ace Glass, Vineland, New Jersey, USA) containing 20 mL of sterile 1× PBS (Thermo Scientific) as collection fluid. Impingers were operated at a constant flow rate of 12.5 L per min using oil-less pumps (Fisher Scientific, Hampton, New Hampshire, USA). Pump performance was monitored using a vacuum pressure gauge

(Cato Western, Tucson, Arizona, USA). Twelve 200-L air samples were taken in succession, i.e., 6 complete evacuations of the DAT over a period of approximately 3.5 h. All samples were maintained on ice until sampling was completed. Thereafter, a 1.5 mL aliquot of each sample was dispensed into a disposable ultraviolet transmissible cuvet (Fisher Scientific, Pittsburgh, Pennsylvania, USA), allowed to warm to 20°C. The amount of rhodamine B dye in each sample was measured using a fluorometer (Turner BioSystems, Sunnyvale, California, USA) equipped with a green optical kit (Turner BioSystems). Results were expressed as raw fluorescence units. Prior to each replicate, the fluorometer was evaluated using a rhodamine B solid standard (Turner BioSystems).

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

$$\frac{d(M_{c,drum}m_a)}{dt} = m_a\{M_{c,in} - M_{c,out}\} \quad \text{[Equation 1]}$$

where:

$M_{c,drum}$ = mass fraction of the target inside drum, kg_{target}/kg_a
m_a = mass of air inside drum, kg_a
t = time, s
m_a = mass flow rate of air through drum, kg_a/s
$M_{c,in}$ = mass fraction of target entering drum, kg_{target}/kg_a
$M_{c,out}$ = mass fraction of target leaving drum, kg_{target}/kg_a

Based on the work of Goldberg et al (25), a DAT is a well-mixed vessel. Therefore, the mass fraction of target leaving the drum is representative of the mass fraction inside the drum and can be stated as:

$$M_{c,drum} = M_{c,out} \quad \text{[Equation 2]}$$

and Equation 1 can be re-written as:

$$\frac{d(M_{c,out}M_a)}{dt} = m_a\{M_{c,in} - M_{c,out}\} \quad \text{[Equation 3]}$$

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

$$M_{c,out}(t) = M_{c,out}(t = 0)e^{-(t\, m_a/m_a)} \quad \text{[Equation 4]}$$

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

$$M_{c,out}(t) = M_{c,out}(t = 0)e^{-(V_{extracted}/V_{drum})} \quad \text{[Equation 5]}$$

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

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

$$M_{c,out}(t = 0) = \frac{M_{c,out}(t_1)}{e^{-(V_{extracted,t_1}/V_{drum})}} \quad \text{[Equation 6]}$$

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

$$M_{c,out}(t) = \frac{M_{c,out}(t_1)}{e^{-(V_{extracted,t_1}/V_{drum})}} e^{-(V_{extracted}/V_{drum})} \quad \text{[Equation 7]}$$

where:

$M_{c,out}(t_1)$ = mass fraction from the first sampled extraction, kg_{target}/kg_a
$V_{extracted,t1}$ = volume of drum air extracted for the first sample, liters
V_{drum} = fixed volume of the drum, liters

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

$$\log_{10} M_{c,out(t)} = \log_{10} M_{c,out(t=0)} - (\log_{10} e/V_{drum})^* V_{extracted}$$

where:

$M_{c,out(t=0)}$ = concentration of rhodamine B at ($t = 0$)
$M_{c,out(t)}$ = rhodamine B concentration at current time
$V_{extracted}$ = the running total of the volume removed
V_{drum} = the total volume in container
e = the base of natural logarithm (Euler's number).

This mathematical representation contained 2 assumptions:

1. There is a linear relationship between ($\log_{10} M_{c,out(t)}$) and $V_{extracted}$;
2. The slope of the rhodamine B regression line was equal to $-(\log_{10} e/V_{drum})$.

If both of these assumptions are true, then, $\log_{10} M_{c,out(t)}$ is a linear function of $V_{extracted}$ with intercept $\log_{10} M_{c,out(t=0)}$ and slope $-(\log_{10} e/V_{drum})$. Thus, the linear relationship between $\log_{10} M_{c,out(t)}$ and $V_{extracted}$ may be used to estimate the concentration of rhodamine B at any time along the regression line. To validate assumption (1), the concentration of rhodamine B ($\log_{10}$) in sequential air samples collected in each of 9 replicates was analyzed using a simple linear regression model using the REG procedure (SAS, version 9.2; SAS Institute Inc, Cary, North Carolina, USA) and the coefficient of determination (R^2) was calculated. To validate assumption (2), the hypothesis that the average slope was equal to the theoretical slope ($\log_{10} e/V_{drum}$) was tested using the Student's t-test.

Results

A total of 10 replicates were attempted. One replicate (number 4) failed because of technical problems that occurred during the procedure. For the remaining 9 runs, least square estimates of the intercept and slope, as well as the coefficient of determination (R^2),

were calculated for each regression line (Table II). R^2 described the proportion of response variation explained for by the linear model and ranges from 0 to 1, with a large R^2 value indicative of a good fit of the linear model. The mean R^2 for the 9 replicates was 0.93 with a standard deviation of 0.07. Overall, the linear regression line explained 93% of the variation in $\log_{10}$ transformed rhodamine B data. The average slope of the 9 runs was not significantly different ($P = 0.1593$) from the theoretical slope (−0.0011). Thus, these data show that the linear relationship between $\log_{10} M_{c,out(t)}$ and $V_{extracted}$ may be used to estimate the concentration of an airborne target in a DAT given known exchange volumes.

Table II. Parameters describing the linear relationship between the concentration of airborne rhodamine B and the volume of air extracted from a dynamic aerosol toroid

Replicate	R^2	Slope	Intercept
1	0.96	−0.0013	3.47
2	0.83	−0.0021	3.40
3	0.96	−0.0030	3.16
5	0.94	−0.0007	3.20
6	0.95	−0.0011	4.07
7	0.80	−0.0007	2.56
8	0.97	−0.0012	4.08
9	0.99	−0.0013	4.72
10	0.98	−0.0016	4.70

Discussion

Successful airborne transmission occurs in 3 basic steps: (i) aerosolization of the infectious agent; (ii) environmentally dependent movement, dilution, and inactivation of airborne infectious particles; and (iii) contact, entry, and replication within a susceptible host (26). From the perspective of prevention and control, the goal is to understand and model the transmission of airborne pathogens in order to design effective counter-measures. Both macro- and micro-level approaches are useful in meeting this objective. That is, field data collected over the course of an outbreak may be useful for modeling the airborne spread of a pathogen within a population (27). Likewise, the basic steps and their components may be evaluated independently under controlled conditions to understand the contribution of each to the process of transmission (18). That is: (i) quantify the rate at which the pathogen is excreted into the environment; (ii) measure the rate of inactivation of the airborne infectious pathogen under specific environmental conditions; and (iii) estimate the likelihood that exposure to a specific dose of the airborne infectious pathogen will produce a response (infection) in an individual host.

This study addressed the third step in this process and, in particular, the specific problem of deriving dose-response curves under experimental conditions in which transmission occurs at concentrations below the threshold of quantification for culture-based methods. In this experiment, a tracer was used to model the behavior of an aerosolized pathogen in a rotating DAT. Tracers (e.g., uranine, rhodamine B, and *Bacillus subtilis* spores) have been used extensively in experimental aerobiology (28). Songer (29) aerosolized rhodamine B dye simultaneously with virus (Newcastle disease virus, infectious bovine rhinotracheitis virus, vesicular stomatitis virus, T3 bacteriophage) to track the physical loss of airborne virus within a DAT. In an experiment of similar design, Hermann et al (30) found no significant difference between the slopes of rhodamine B dye and porcine reproductive and respiratory syndrome virus RNA detected by quantitative PCR, i.e., the concentrations of rhodamine B and viral RNA declined in the DAT at the same rate. Under the conditions of this experiment, the fact that the theoretical line and the experimental line were not significantly different provided evidence that physical loss did affect the outcome of the tracer values. Thus, rhodamine B concentration has been shown to reflect target pathogen concentrations under conditions similar to those reported here.

The physical parameters and experimental conditions of this study merit discussion. This experiment was conducted in a 400 L DAT rotated at 4 RPM. However, a variety of DAT sizes and rotation speeds are reported in the literature, for example, 140 L (29), 1000 L (31), and 2500 L (32). A review of the literature found no evaluation of the effect of DAT dimensions, volume, and rate of rotation on the behavior of suspended particles. Therefore, it would be of value to confirm the results reported here using the described methodology.

In this experiment, the environmental conditions were designed to preserve target pathogen infectivity. In particular, the −4°C temperature at which the DAT was maintained would be expected to preserve the infectivity of a target pathogen indefinitely. At temperatures above freezing, the slope of the airborne pathogen would diverge from the slope of the rhodamine B. Therefore, the inactivation of the target pathogen over time would need to be accounted for in the estimation of the airborne pathogen concentration. This is not an insignificant consideration because the rate of airborne pathogen concentration inactivation is affected by isolate (26,27), the suspension medium (32,33), temperature (29,34), and relative humidity (30,35). Therefore, it is preferable to avoid this complication by maintaining the DAT at temperatures below 0°C.

In dose-response studies, the CSTR model solves the problem of estimating the exposure dose when the concentrations of airborne pathogens are at microbiologically unquantifiable levels.

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

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