College of Engineering

A Quantitative Microbial Risk Assessment Model for Legionnaires' Disease: Animal Model Selection and Dose-Response Modeling

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Abstract

Legionnaires' Disease (LD), first reported in 1976, is an atypical pneumonia caused by bacteria of the genus *Legionella*, and most frequently by *L. pneumophila (Lp)*. Subsequent research on exposure to the organism employed various animal models, and with Quantitative Microbial Risk Assessment techniques, the animal model data may provide insights on human dose-response for LD. The present report focuses on the rationale for selection of the guinea pig model, comparison of the dose-response model results, comparison of projected low-dose responses for guinea pigs, and risk estimates for humans. Based on both *in vivo* and *in vitro* comparisons, the guinea pig (*Cavia porcellus*) dose-response data were selected for modeling human risk. We completed dose-response modeling for the β-Poisson (approximate and exact), exponential, probit, logistic and Weibull models for *Lp* inhalation mortality and infection (end point elevated body temperature) in guinea pigs. For mechanistic reasons, including low-dose exposure probability, further work on human risk estimates for LD employed the exponential and β-Poisson models. With an exposure of 10 Colony Forming Units (retained dose), the QMRA model predicted a mild infection risk of 0.4 (as evaluated by seroprevalence) and a clinical severity LD case (e.g., hospitalization and supportive care) risk of 0.0009. The calculated rates based on estimated human exposures for outbreaks used for the QMRA model validation are within an order of magnitude of the reported LD rates. These validation results suggest the LD QMRA animal model selection, dose-response modeling, and extension to human risk projections were appropriate.

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Microbial risk assessment, dose response assessment, Legionella pneumophila, Legionnaires' disease

1. INTRODUCTION

The first goal for developing the Quantitative Microbial Risk Assessment (QMRA) model for Legionnaires' disease (LD) involved assembling and evaluating data to estimate human risk from animal model dose-response information. Next, since the information was sufficient to proceed, we develop the QMRA model and validating it by comparing calculated human LD risk estimates to reported LD outbreak rates. The LD QMRA model may have utility in estimating the health risk to people resulting from exposure to aqueous aerosols produced by sources such as cooling towers, whirlpool spas, showers, and other water sources that contain *Legionella*. Prior reports suggest that the infective dose-response for LD has not been established and many factors need to be considered in understanding LD risks [(1)]. QMRA techniques provide the framework within which the analyses and linkages of the hazard and exposure data may be completed. Figure 1 shows LD QMRA project in three parts, as divided for model development and publication. The current manuscript focuses on Part I and covers primarily the animal model selection, dose-response modeling and initial considerations of human risk based on the animal dose-response data. Parts II and III, covering exposure assessment for human exposures during three outbreaks reported in the literature, and evaluation of human risk projections, respectively, are reported in a dissertation [(2)] that is available as a PDF file, and in other manuscripts [(3, 4)]. Full details on the three parts are beyond the scope of a typical journal manuscript. Thus, this current report focuses on the animal model selection, and summarizes supporting aspects from the exposure assessment and model evaluation.

Bacterial exposures are quantal. The common expression for bacterial quantities in dosing is in colony forming units (CFU) where each colony on a culture plate represents one viable (and culturable) organism and thus a measure of the number of bacteria. In this manuscript, the low dose results will show what might be taken as a fractional CFU exposure, but this is not the correct interpretation. For example, an exposure dose of 0.1 CFU means one person in ten would receive a dose of 1 CFU.

2. METHODS

2.1. Animal Model Selection

For *Legionella pneumophila*, available evidence suggests that, from the animal inhalation exposure models reported in the literature, guinea pigs (*Cavia porcellus*) are the most appropriate model choice for human risk extrapolation. Table 1 summarizes *in vivo* Legionella inhalation data for the range of animal models. Data from intraperitoneal injection, nasal or tracheal instillation studies were set aside as less relevant for an inhalation risk assessment. The Lewis rat data shown in Table 1 do not resolve the rat as suitable or not suitable, but additional information (paragraph below) suggest the rat is relatively resistant and does not develop LD similar in severity and effect to that in humans. Other species for which we found inhalation exposure data, summarized in Table 1, appear less susceptible to Legionella than do guinea pigs. Table II provides a summary of the broader rationale for the guinea pig model selection and Table III provides an analysis of *in vitro* and mechanistic aspects for the broader range of animal model data. The justification for the guinea pig model is largely from the *in vitro* data summarized in Table III. Uptake, survival, and growth rates for *Lp* appear more similar in human and guinea pig macrophage lines than between human and other species macrophages for which data were located. Due to a lack of data for comparisons, this analysis currently neglects subsequent stages of cell-mediated and humoral immune system responses [5, 6]. As noted in Table III, published literature suggests that most mouse strains and other animal models evaluated are relatively resistant to Legionella infection. Studies [7-9] showed the rat (Sprague-Dawley and Lewis strains) is a resistant model compared to guinea pigs, and found more similarities of LD in guinea pigs and humans that in the rat and humans. Aerosol exposure data for Legionella and LD development in the rat model were limited [9] with respect to dose groups and dose determination, which limits utility for dose-response modeling in a QMRA project. Given these reasons, the rat model was not considered further in the QMRA. The limited non-human primate data [10, 11] suggest relative resistance to LD, were not in a form for dose-response modeling, and thus were not considered further for the QMRA model development.

We did not apply risk assessment techniques to statistically explore the influence of different animal models' on human risk projections [12]. The *in vivo* and *in vitro* data support the guinea pig selection, and in our opinion demonstrate that the other available animal models are less suitable. Additionally, as shown in the subsequent LD QMRA validation, the guinea pig data appear to be satisfactory for predicting human risks.

2.2. Dose-Response Data

We conducted a literature search for published studies on inhalation studies for Legionella in animal models, with the emphasis on guinea pig data for the reasons outlined above. Table IV lists reports that provide data potentially suitable for dose-response analyses. Further review narrowed the list to the studies we selected for further evaluation. The selected studies all used similar exposure methods to deliver 5 micrometer aerosol, provided verification of dosing, evaluated the dose retained in the animal lungs, and detailed the dose group sizes and responses. Human exposure to Legionella results in a range of responses, from apparently silent development of antibodies to Legionella (seroconversion), to mild fever and recovery, to clinical severity illness requiring medical care, to mortality [13, 14]. Thus, data to evaluate mild infection as indicated by fever, and severe infection as indicated by animal mortality were both of interest.

Infection has been likened to a battle between the adaptive bacteria's mechanisms for survival and replication in a host organism, and the host organism's antimicrobial defense mechanisms [14]. The range of effects then likely overlap, with subclinical infection indicating the stage where host defenses succeeded, but possibly would not have in a slightly more susceptible host or with more virulent bacteria, or other slight change. Clinical infection may indicate a much more serious battle, usually requiring medical support, and mortality indicates the battle lost by the host. Projections of subclinical infection risk may thus be informative supplements to the clinical infection and mortality risk estimates. For mild infection dose-response modeling, one report [15] was available and suitable. For mortality analyses, more reports were available, but following review, 4 were used in preliminary analyses [11, 16-18], with one [16] selected for more detailed projections.

2.3. Dose-Response Modeling

Haas [19] described methods for quantitative microbial risk assessment and low dose risk modeling. Haas et al. [20] subsequently further describe the derivation and application of dose response modeling for microbial data. Two models – the exponential and β-Poisson derive from biological mechanistic considerations. That is, the host must receive at least one organism and the microorganisms may undergo decay in viability or loss via host defense and risk does not exceed the probability of exposure to at least one microorganism. The decay/defense may be represented by constants in the exponential model or by distributions (e.g., the beta distribution in the β-Poisson model). Haas et al. [20] provides further discussion and justification for model selection. Other commonly used dose-response models have empirical bases and these include the logistic, probit and Weibull models. Figure 2 shows the equations for the models used.

The commonly used β-Poisson model -

$$P_1(d) = 1 - \left(1 + \frac{d}{\beta}\right)^{-\alpha}$$

where P_1 = the response at dose d and α and β represent model parameters - is based on an approximation that holds only when the value for β is >>1 and α is << β. [20-22] Teunis [23] demonstrated that, at low doses, (even with alpha and beta values in the acceptable range), the approximate β-Poisson model predicts risk exceeding the probability of exposure, and that is not plausible. However, the exact solution for the β-Poisson relationship is more computationally challenging.

The dose-response modeling, except for the exact β-Poisson work, was done with Microsoft Excel®, using maximum likelihood techniques and the Excel feature Solver for numeric optimization. The exact β-Poisson model analyses were completed using Mathematica® software. We tested the Excel Solver optimization convergence by rerunning the optimization with the parameters reset to either direction from the initial optimum set, but did experience inconsistencies in convergence with the data sets we used. The fit of the model to the data was judged satisfactory if the likelihood value was less than the critical Chi

square value, with the degrees of freedom equal to the number of dose groups minus the number of model parameters. These fitting techniques and goodness of fit evaluation methods have been described elsewhere [20, 24]. We also graphically examined the low-dose projections of the models as part of the model evaluation. We proceeded with the β-Poisson and exponential models due to their low-dose linearity with exposure probability and their mechanistic basis. Given the subsequent findings of good agreement between projected risks and reported risks in our QMRA validation stage [4] as summarized the following section (2.4) of this current report, we did not conduct further work on the model selection for the LD QMRA. Future research, however, could consider application of more structured model selection and comparison approaches [25].

2.4 Human Exposure Assessment and LD QMRA Model Evaluation

In order to evaluate the LD QMRA model, we needed information on human exposures and the related rates of LD. Despite extensive searches, we did not locate reports where the exposures to Legionella were quantitatively evaluated at a time relevant to LD cases' exposure. We therefore developed and applied approaches for estimating Legionella exposures for a whirlpool spa related outbreak [26-29], and two natural hot springs spa outbreaks [30-32]. These were selected since the reports were relatively rich in details to support an exposure assessment compared to other published outbreak reports. Further details on the selection and exposure assessment are provided elsewhere [2, 3]. Exposure estimates for the whirlpool spa outbreak used aerosol generation information, assumed *Lp* content in water (from published reports), estimated bacterial content of aerosol, time of workers in the building exposure zones, and two distance zones of workers from the whirlpool, using a two zone model. The distribution of estimated exposures was then calculated via Monte Carlo simulation.

Exposures for the two hot spring spa outbreaks were estimated from reported Legionella in water concentrations, water to air bacterial partitioning coefficient, and estimated time spent in hot spring environment. The distributions of estimated exposures were generated via Monte Carlo simulation.

The respective exposure distributions were fed into the dose-response model using Monte Carlo simulation. The resulting estimated risk distributions were then compared to the reported rates for the outbreaks [2, 4].

2.5 Uncertainty Analyses

Our LD QMRA project plan involved assembling and applying available information to estimate human risks and validate the findings. A part of that plan included identifying where further work would reduce the uncertainties and improve the utility of the LD QMRA. With the stochastic calculations and software used, sensitivity analyses are straightforward for those aspects. Such sensitivity analyses, as commonly applied, cover the sources of variability and this may not reflect the sources of uncertainty. Some data may be highly variable but quite reliable, and other data may show little variability but be rather speculative and a source of significant uncertainty. For the LD QMRA, we provide primarily a qualitative analysis of the key uncertainties and their magnitude in this current report. We reported the sensitivity analyses for the exposure assessment and validation stages elsewhere [2-4]. A more structured quantitative uncertainty analysis plan was not included in the completed research project's scope.

3. RESULTS

All the studies shown in Table IV, except two [10, 15] reported 100% infection in the lowest dose groups. The one study [10] may not have provided adequate follow-up time after dosing for infection to manifest in the lowest dose group; the report indicates follow-up was 2 to 3 days post exposure. The data of Muller et al. [15] showed the guinea pig rectal temperature (an indicator of response to infection) in the lowest exposure group did not rise above baseline until day 6 post exposure, but was above baseline by day 3 for a higher dose group. For humans, the generally recognized incubation period is 2 to 10 days [13], but perhaps longer since one report listed cases at up to 19 days post exposure [28]. The medical literature on infectious diseases suggest that incubation periods for several diseases are longer at lower doses [14]. The other data sets with 100% response for signs of infection in the low dose group are not as informative in dose-response modeling for infectivity. However, those data are not inconsistent with Muller's [15]

findings since the low dose groups and responses were in the range of Muller's higher dose groups. Fitzgeorge [11] and Baskerville [10] estimated the guinea pig inhaled dose based on exposure period, inhalation rate (0.15 liters/minute) [33] and 50% retention of the aerosol [34]. Berendt [18] used the same approach and the same assumptions. Fitzgeorge [11] comments (but did not publish the raw data) that the calculated dose corresponded well to viable counts recovered from lung macerates of guinea pigs sacrificed immediately following exposure.

Using the data from Muller [15] led to estimates for the doses for 50% and 1% infection rates ($ID_{50\%}$ and $ID_{1\%}$), as shown in Table V. All models listed passed the goodness of fit test based on comparison of the likelihood ratio to the critical values of Chi square (X^2) at the 95% level using methods as described previously [20, 24]. Table VI shows the goodness of fit results and parameter values for the models applied to the infectivity data. None of the models gave a significantly improved fit according to the Chi square evaluation using the method described elsewhere [20, 24]. The approximate β-Poisson and exact β-Poisson models gave the same results, with the approximate form then used in further work.

For mortality , there is a wider selection of reported data available for consideration [9-11, 17, 18, 35, 36]. However, not all of these data proved suitable for dose response analyses. Aspects that limited their utility included: a) no responses other than 0 or 100%, b) non-monotonic responses, c) inadequate documentation of the number of animals or d) inadequate details on dosing. For the study showing non-monotonic response [37], the report was also difficult to decipher with certainty with respect to the dosing of the low dose, dose group sizes and responses. However, interpreting the repost as well as possible, the model fits were not satisfactory. The exponential model results were typical of the other model fits, with the likelihood ratio of 66 exceeding the critical Chi square of 7.8. Thus, we focused on comparative analyses of those data sets listed in Table VII, with the data of Baskerville [16] selected for the subsequent risk projections and low dose extrapolation. Table VII shows the projected $LD_{50\%}$ and $LD_{1\%}$ results, the fitted model parameters, and the model goodness of fit test results.

Combining the data sets listed in Table IV for analysis (by arraying the studies dose levels in numeric order, with the respective responses) led to results that did not satisfy the goodness-of-fit criteria. Without further research, this combined data set approach did not seem prudent to pursue, since the studies involved differing bacterial strains as well as differing mixes of guinea pig strain, maturity and sex.

3.1. Low Dose Extrapolation

Low dose extrapolation is an important consideration in model selection. Models may give similar results in the range of available data, yet the main interest often is at lower doses. Then, the shape of the extrapolated curve has major impact. For analysis of the Muller [15] infectivity data, the low dose predictions for the models considered follow in Table VIII and are shown in Figure 3. The mortality risk projections shown in Table IX are based on the data of Baskerville [16], and are shown graphically in Figure 4.. Note that the results from the Exponential and β-Poisson models agree quite well, including for the low-dose projections. At low doses, the logistic and probit models rapidly diverge from the other models and are notably sub-linear with dose. On the mechanistic consideration that risk is limit by exposure probability, and with attenuation of dose either as a fixed value or as a distribution, the exponential and β-Poisson results seem to be the most appropriate for low dose risk predictions from these data sets. This is in part based on our interest in evaluating the conjecture that risk at low dose for Legionella still relates to exposure probability. The empirical models' results are arguably less relevant than the more mechanistic models, and we therefore did not use the empirical model results further in our current LD QMRA work. As reported elsewhere [2], the evaluation of the QMRA model demonstrates the exponential and β-Poisson projections fit reasonably with reported human risks.

3.2. Interspecies Susceptibility to Legionella Infection

A relevant question is - how well does the guinea pig model for Legionella apply to human risk projections? The guinea pig model has been widely used for *Lp* inhalation studies and other animal model systems have also been studied. No studies are known which offer direct comparative data on human versus other species response to *Lp* inhalation. Different *Legionella* species and strains used by different investigators complicate animal model comparisons; virulence may differ by species and strain

[11, 38]. Following inhalation exposure, rats and mice showed relative resistance to *Lp* [10, 11, 16]. Rhesus monkey and marmosets develop symptoms of LD similar to those seen in guinea pigs [10, 16]. The monkeys and marmosets appeared to be somewhat more resistant to infection than were the guinea pigs [16], as the calculated inhaled dose for the monkeys was 6×10^6, and marmosets 5.5×10^5 CFU, with a milder course of disease, but at "relatively lower doses." The marmosets [16] showed one of four moribund at sacrifice following a dose 4.5×10^5 CFU. The guinea pig predicted 25% mortality (using the data of Baskerville [16]) is at 4.8×10^3 CFU. Comparing these then shows a dose approximately 100 times higher for an equivalent response in the marmosets, and this implies a marmoset resistance to mortality approximately 100 fold higher. The Rhesus monkey data showed infection of 7 of 8 at 10^6 CFU, with no mortality by time of sacrifice. The Muller [15] guinea pig infectivity data predicts 7 of 8 responding at 35 CFU, if Rhesus monkey sensitivity equals that of guinea pigs. However, the limited Rhesus response data translates to an apparent sensitivity difference (compared to guinea pigs) of approximately 3×10^4. Review of comparative data for animal models and different bacteria suggests that a wide range of relative susceptibility may be expected. For anthrax spores, which may include wild and weaponized materials, published $LD_{50\%}$ results range from 4×10^3 for Cynomolgus monkeys to 3×10^6 for pigs [39-42]. Thus, selection of an appropriate animal model appears to be a key aspect of QMRA.

Due largely to the findings shown in Tables II and III, the guinea pig data are presumed to apply directly to predict human risk. The available data give no rationale to adjust for either greater or lesser sensitivity and our subsequent LD QMRA validation findings [2, 4] support this decision. One of the studies cited in Table III of particular relevance [43] evaluated intracellular production of protease in isolated guinea pig and human alveolar macrophages and showed similar growth rates at similar dose levels, with similar protease production. Within 24 hours following dosing of the cultures, *Legionella* counts increased by 2 to 3 orders of magnitude in both species' macrophages. This is relevant since *Legionella* are known to replicate in macrophages *in vivo* in both species, which is a key factor in the organism's pathogenicity.

3.3. Dose Scaling

With a given dose of infectious organisms, does response to pathogens vary with animal model body mass or other body scale metric? If so, perhaps dose scaling by body mass or inhalation volume or

another metric is appropriate. However, we are not aware if dose scaling for inhalation pathogens is fully resolved as either appropriate or inappropriate for quantitative microbial risk assessments. This aspect of QMRA has clear differences from radiation and chemical risk assessments where scaling is routinely addressed. For chemicals, the received dose is the total ever available to cause toxicity. However, pathogens have the potential to replicate in the host organism. Thus, there is a much different aspect to consider in microbial risk assessment. That aspect is the ability of the initial inoculum, possibly a single viable and virulent bacterium, to survive, multiply, and cause disease in the host.

An intracellular pulmonary pathogen such as *Lp* must succeed in several (arguably) probability-based events to cause infection in a host species. If these probability requirements are met to a sufficient frequency, then a certain probability of infection manifests. Several of these probability based events are: viability and virulence of the particular *Lp* organisms, deposition of aerosol containing the *Lp* in the pulmonary tract, especially the alveolar region and uptake by a macrophage, replication of *Lp* in a compliant macrophage, and lysis of the host macrophage and infection of subsequent compliant macrophages (a “chain reaction”).

For Legionella, the late stages of an unchecked infection include the release of toxins, proteases and associated inflammatory responses manifesting as clinical disease [(14)]. The total probability (given each event is independent) is the product of the individual events’ probabilities. We suggest that this infection probability is not dependent on the total lung surface area or inhalation volume and thus does not scale with body weight, or lung volume. The key aspect for LD is the number of organisms deposited and available for subsequent uptake by and replication in compliant alveolar macrophages. Since we did not find data to the contrary, we see no reason at this time for major differences in the surface density (number per square centimeter of lung surface) of resident alveolar macrophages (AM) in guinea pigs, other animal models, and humans. Data suggest approximately one resident AM per alveolus in humans [(44)]. Dose scaling for an intracellular pulmonary pathogen such as Legionella would appear, with this line of reasoning, to be inappropriate. Our subsequent LD QMRA validation findings [(2, 4)] suggest this reasoning is appropriate.

3.4. Risk Projections for Humans

Palm et al. [45] and Kliment [46] show similar deposition patterns in pulmonary regions for 5 micrometer particles in guinea pig versus human systems. The animal model reports (see Table IV) did not provide analysis of the initial 5 micrometer aerosol deposition patterns in the animal pulmonary tracts. Clearance half lives for insoluble particulates are on the order of days and longer for humans and it is of the same magnitude for guinea pigs [47, 48]. These clearance rates suggest negligible removal of the delivered dose for short-term (minutes to hours) exposures used in the aerosol dosing experiments. For LD, deposition in the alveolar region is relevant [14], but given the scope of work for our total project and the animal data constraints, we did not further address potential differences in pulmonary tract deposition.

Tables VIII and IX present the low dose projections for infection risk and mortality risk for the models tested, with Table VIII providing the projections for infectivity. Note that the low-dose extrapolated risk is linear with dose for the exponential, approximate beta-Poisson and exact beta-Poisson models. Thus, estimates of infection risk for humans from the exponential model are, for a risk of 1 case in 1000 so exposed, approximately 2 CFU and for a risk of 1 case in 10^6 so exposed, approximately 1 CFU inhaled by 1 person in 500. The mortality projections from the exponential model indicate a risk of 1 case in 100 exposed at 117 CFU retained dose, 1 in 1000 at approximately 12 CFU, and 1 in 10,000 risk at a retained dose of approximately 1 CFU. These initial projections presume a virulent strain and human susceptibility equivalent to that of Guinea pigs. These assumptions were tested in part three of our work, with the evaluation results summarized elsewhere [49] and reported in more detail in a subsequent manuscript [4]. The findings are also currently available in a dissertation [2].

3.5. Summary of Outbreak Exposure Assessment and Risk Comparisons

For the whirlpool spa, the predicted zone 1 exposures as retained dose were a mean of 10 CFU with a 95% range 1.3 to 34, and the predicted zone 2 exposures as retained dose were a mean of 7 CFU with a 95% range 1.3 to 19. For the two hot springs spa outbreaks, the predicted exposures were for the one

outbreak a mean 47 CFU with a 95% range 24 to 84 and for the other a mean of 2.3 CFU with a 95% range of 1.1 to 4.1.

These corresponding air concentration estimates (that led to the retained dose values given in the paragraph above) are in the general range of air concentrations reported (2 to 190 CFU/m3) for Legionella concentrations in air near showers and aerated faucets supplied by Legionella contaminated water [50-53], as summarized previously [3]. Note that these cited air concentrations did not tie to cases' actual exposure and were not contemporaneous to outbreak exposures and subsequent disease development.

The respective exposure distributions were fed into the dose-response model using Monte Carlo simulation. The resulting estimated risk distributions were then compared to the reported rates for the outbreaks. The confidence intervals of the predicted risks generally overlap the confidence intervals on the reported rates of LD, or miss by less than 10× [2, 4]. This suggests that the model is generally valid, for the animal model selection, the dose-response model application, and the *a priori* decision on no dose scaling and no intra-species adjustments.

3.6. Uncertainty Analyses

As mentioned earlier, our original project plans and work scope did not include a comprehensive quantitative uncertainty analysis. To a large degree, the QMRA validation results suggest that a more extensive uncertainty analysis might not be a reasonable research priority at present. The qualitative uncertainty analysis and sensitivity analyses completed and summarized may give sufficient insights on the current strengths and limitations, and show where additional research would be most productive in improving the LD QMRA. Table XI summarizes the factors used in the QMRA project, largely following the flow shown in Figure 1. The table provides an analysis of the major uncertainties in the LD QMRA model, but with generally qualitative ranges for the impact.

4. DISCUSSION

The prevalence of Legionnaires' disease in humans is considerable, even according to incomplete data due to probable underreporting [54]. *Legionella* bacteria often colonize and can amplify in various aqueous systems, including the circulating water in cooling towers, whirlpool spas, natural hot springs, and hot water distribution systems. Aerosols from a broad range of *Lp* contaminated systems have been implicated as the sources in outbreaks and sporadic cases of Legionnaires' Disease. Estimates of human mortality following infection vary, but often are in the range of 10 to 15 % [13]. Given the prevalence of aqueous aerosol systems, *Lp* contamination of them represents significant potential for infection and subsequent morbidity and mortality. The currently reported work suggest that *Lp* has the potential for significant low dose infectivity in guinea pigs, and by using risk assessment techniques the guinea pig data may be extended to estimate the LD risk to humans following inhalation exposure.

4.1. Strengths of the Current Study

The dose response modeling demonstrates a good fit of the models to key data sets. There is also a consistency between the models in the range of observations, but divergence by the empirical models for low dose extrapolation.

The available *in vivo* and *in vitro* data reviewed suggest that the guinea pig model is the most appropriate one of those available for human risk projections.

The subsequent LD QMRA evaluation results show good agreement between predicted and reported rates of disease. The suggests both the animal model selection and the dose-response models used were appropriate, as were the *a priori* decisions on no dose scaling and no inter-species sensitivity adjustments.

4.2. Limitations of This Work

1. Limited data for dose-response modeling on infectivity. Infection is a key concern as it indicates the initial immune response to a replicating bacterial strain, yet only one report had data suitable for dose-response modeling on infectivity. The data used for the infectivity dose-response modeling [15] compares

reasonably with other low dose studies on guinea pigs, and this gives some additional confidence to the guinea pig infectivity dose-response analysis. Since the QMRA results for sub-clinical infectivity fit well with reported rates, the limited data appear adequate.

2. Broad applicability to humans. Legionella are adaptive organisms, with multiple virulent species in the genus, and multiple strains within specific species. Virulent Legionella exhibit a variety of mechanisms for intracellular growth [55] which interact in possibly different ways with different animals and man. The current state of knowledge for Legionella is extensive and growing, but the comparative immunology may not give inarguable proof that a particular animal model as the ideal choice as a human surrogate. The comparative data on macrophages for humans and guinea pigs is important, but the subsequent stages of immune system response undoubtedly are also important, and so additional immune responses need further consideration in the LD QMRA. Our work on the LD QMRA model validation as summarized in results, and reported elsewhere [2, 4] substantially augments and substantiates the *in vitro* and *in vivo* data that suggests the guinea pig data provide an adequate base from which to estimate human LD risks.

4.3. Work Remaining

1. There are recognized risk factors [56-60] that lead to a range of human intra-species sensitivity. Known main factors include age, gender, smoking status, obesity, recent prior infections to other organisms, and immune system competence. Further research would be needed to resolve the impact of these risk-modifying factors.

2. Many other factors need to be considered in a full assessment of human risk following Legionella exposure. One issue is the transport and retention of virulence in aerosol form. In aerosols, Legionella may retain viability longer than ability to grow in standard culture [61]. A recent study showed, for a fresh aerosol in a chamber, liquid impinger sample collection and FISH determination gave several orders of magnitude higher counts than did filter collection and analysis by culture [62]. The existing data using culture to evaluate Legionella survival in aerosols [63-65] may have significant limitations, and additional research on Legionella viability in aerosols is needed. Another issue is the relative virulence of Legionella, by strain, species and by growth stage or niche adaptation. The ecologic adaptation aspects also include consideration of virulence shifts in Legionella with adaptation to intracellular growth in

protozoa, and the role of protozoan vesicles containing Legionella in aerosol dispersal, viability and infectivity. This list is illustrative of on-going research needs for a LD QMRA and may not be comprehensive.

3. The uncertainty analysis and the sensitivity analyses completed have identified areas for further research to reduce total model uncertainties. The validation suggests the LD QMRA model predicted risks are within an order of magnitude of reported outbreak rates, but this is based on estimated exposures using data with significant (order of magnitude) uncertainty. Thus, investigations to reduce the uncertainty in the exposure assessments and the subsequent validation's adequacy remain to be completed. A full quantitative uncertainty analysis is an area for future work, but is not likely to shift the currently identified research areas.

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Table I. *In Vivo Legionella pneumophila* comparisons
All by inhalation, as retained dose in lungs

Species	$LD_{50\%}$ (CFU)
Guinea pig	8,000 – 17,000 [11, 16, 17]
Porton mice	>25,000 [16]
Lewis rats	>1200 [8]
Marmosets	450,000 [16]
Rhesus & Cynomolgus monkeys	>1,000,000 [16]

A Colony Forming Units (CFU) from microbial culture represents a culturable bacterium, so CFU represents the count of culturable bacteria, but assumes each counted colony grew from a single organism.
Data on infectious dose (e.g., $ID_{50\%}$) in different species are too sparse for a comparison table.

Table II. Summary of the Basis for Guinea Pigs as the Preferred Model for Human Risk Prediction
The details supporting this summary table are provided in Table III and in the text discussion.

Factor	Comments and References
Similarity in Nature and Course of Disease	Development of fever, subsequent pneumonia, and mortality [10]
Respirable Aerosol Deposition and Retention	Both species show approximately 50% deposition of respirable range aerosols in the pulmonary tract, with similar fractional regional deposition. [45, 46]
Alveolar Macrophage Uptake and Replication	The *in vitro* rate of uptake is similar in both species. The in vitro replication fraction and rate of replication is similar in both species. [43, 44, 66]
Alveolar Macrophage Bactericidal Mechanism Responses	The reactive oxygen mechanism is subverted in both species. [67-70] The reactive nitrogen species mechanism takes induction in both species, and is more strongly resident in more Legionella resistant animal species for which such data are available, such as rats and most mouse strains. [71-74]

Table III. *In Vitro* Endpoint and Mechanistic Comparisons for *Legionella pneumophila*

Mechanism or Endpoint	Discussion
Macrophage Reactive Oxygen Intermediates and Oxidative Burst	Guinea pigs – subverted by Legionella as the usual fusion with lysozomes and oxidative burst does not occur [67] in guinea pig peritoneal macrophages [75] Humans – the mechanism is subverted by Legionella [43] [68] [69] [70] Pigtail monkeys – the mechanism is remains operative [76]
Macrophage Reactive Nitrogen Intermediates	Guinea pigs – minimal production [71] Humans – minimal production [72] and minimal bactericidal role [73] in monocytes and slow (days) induction [74] Murine – production varies by strain of mouse, and correlates with observed virulence [71] Non-human primates – not known (information not located for Lp), active for simian infection with Simian Immunovirus [77]
Fraction of bacterial dose surviving macrophage phagocytosis and replicating	Guinea pigs – most survive (>90%) post phagocytosis [43] Human – most survive (90% to 100%) post phagocytosis [66] and replicate 3 to 5 log in 2 to 3 days [44] [66] Mice – Most inbredmouse stains are resistant to L. pneumophila. [76, 78-80]. Rat – apparently resistant to Legionella replication and survival [81, 82] Pigtail monkeys - showed low phagocytosis, approximately 1% of the inoculum, [83] with 2.5% to 5% survival [76, 83] post phagocytosis. The surviving fraction replicates 2 log in 4 days [83] Cynomolgus monkeys - at high bacteria to macrophage ratio (100:1) demonstrate slow uptake (5% of cells at 3 hours) but significant intracellular growth of the surviving fraction [67, 84] Similarities in survival, growth rates and protease production in guinea pig and human alveolar macrophages *in vitro* and human virulence well-modeled in guinea pig [68]

Table IV. Comparison of Infectivity Data from Inhalation Exposure Studies of Guinea Pigs

Study	Dose (CFU)	Finding
Muller [15]	12	Estimated 50% infection
Twisk-Meijssen [37]	4* to 12*	100% with fever by day 7
Berendt [18]	129	All (16 of 16) developed fever
Breiman [17]	200	100% developed fever
Fitzgeorge [11]	200	10/10 had fever by days 3 to 4
Baskerville [16]	2400	12/12 developed fever
	200	0% with fever**
Meenhorst [35]	2500	8/8 developed fever
Jepras [38]	8000	100% at days 2 to 6 developed fever
Davis [9]	12000	100% had symptoms of illness

*Estimated from retention in higher dose groups. **At 2 to 3 days post exposure, length of follow-up not clear

Table V. Predicted Infectious Dose-Response, with 95% Interval for the Response (as % Response)

Model	$ID_{50\%}$ CFU	$ID_{1\%}$ CFU
Exponential	11.7 (28 –78)	0.17 (0.5 – 2)
Approximate beta-Poisson	11.5 (30 – 80)	0.17 (0.5 – 4.1)
Exact beta-Poisson*	11.5	0.17
Weibull	11.6 (22 – 76)	0.17 (0.02 – 12)
Probit	9.2 (23 – 75)	0.90 (0 – 18)
Logistic	8.7 (20 – 80)	0.64 (0.01 – 15)

* confidence intervals not calculated. Data of Muller [15].

TABLE VI. Model Goodness of Fit and Parameter Values

Mullet et al. [15] Guinea pig infectivity data

Model	Goodness of Fit and [Critical Chi Square]	Parameter Values
Approximate beta-Poisson*	0.58	β 1700
	[5.9]	α 102
Exponential	0.58	r 0.06
	[7.8]	
Weibull	0.58	q^1 0.06
	[5.9]	q^2 1.0
Probit	0.30	q^1 9.2
	[5.9]	q^2 1.0
Logistic	1.5	q^1 3.8
	[5.9]	q^2 1.76

* The exact and approximate beta-Poisson results were equivalent to 2 significant figures.

Table VII. Estimated Lethal Doses in Guinea Pigs, as CFU Retained in Lungs, with Fitted Model Parameters and Model Goodness of Fit Statistics.

Data Source and Model	$LD_{50\%}$	$LD_{1\%}$	Fitted Model Parameters	Goodness of Fit Likelihood (Critical X^2)
*Baskerville [16] Exponential	8,000	117	$r = 8.7\times 10^{-5}$	2.45 (7.81)
Approximate beta-Poisson	7,500	116	$\beta = 5.2 \times 10^{10}$, $\alpha = 4.8 \times 10^{6}$	2.47 (5.99)
Weibull	8,500	270	$q_1 = 2.3 \times 10^{-6}$, $q_2 = 1.4$	0.303 (5.99)
Probit	3,200	2,000	$q_1 = 3.19 \times 10^{3}$, $q_2 = 2.1 \times 10^{-1}$	0.000 (5.99)
Logistic	3,200	1900	$q_1 = 6.89 \times 10^{1}$, $q_2 = 8.5$	0.000 (5.99)
*Fitzgeorge [11] Exponential	10,700	140	$r = 6.47\times 10^{-5}$	2.34 (7.81)
Approximate beta-Poisson	11,600	170	$\beta = 1.8 \times 10^{12}$, $\alpha = 1.1 \times 10^{8}$	2.37 (5.99)
**Breiman [17] Exponential	16,900	247	$r = 4.11 \times 10^{-5}$	5.78 (7.81)
Approximate beta-Poisson	16,600	243	$\beta = 3.9 \times 10^{10}$, $\alpha = 1.5 \times 10^{6}$	5.78 (9.49)
*Berendt [18] Exponential	108,000	1580	$r = 6.4\times 10^{-6}$	6.42 (7.81)
Approximate beta-Poisson	107,000	1570	$\beta = 9.8 \times 10^{10}$, $\alpha = 6.3 \times 10^{5}$	6.42*** (5.99)

* Estimated retained dose in animal lungs, ** Assay of retained dose in animal lungs, *** Not a satisfactory fit.

Table VIII. Summary of Guinea Pig Low Dose Infection Risk Predictions (as % Response) Modeled on Data of Muller et al. [15].

Dose	MODEL				
CFU	Exponential	beta-Poisson (Approximate)	Weibull	Probit	Logistic
10	45	45	45	53	56
1	6.0	5.9	5.8	1.4	2.7
10^{-1}	6.0×10^{-1}	6.1×10^{-1}	5.9×10^{-1}	3.7×10^{-4}	3.9×10^{-2}
10^{-2}	6.0×10^{-2}	6.1×10^{-2}	5.9×10^{-2}	6.6×10^{-10}	6.7×10^{-4}
10^{-3}	6.0×10^{-3}	6.1×10^{-3}	5.9×10^{-3}	0	1.2×10^{-5}
10^{-4}	6.0×10^{-5}	6.1×10^{-4}	5.9×10^{-4}	0	2.0×10^{-7}
10^{-5}	6.0×10^{-5}	6.1×10^{-5}	5.9×10^{-5}	0	3.6×10^{-9}
10^{-6}	6.0×10^{-6}	6.1×10^{-6}	5.9×10^{-6}	0	1.1×10^{-12}

Table IX. Summary of Guinea Pig Low Dose Mortality Risk Predictions (as % Response)

Modeled on the data of Baskerville et al. [10]

Dose	Model				
CFU	Exponential	beta-Poisson (Approximate)	Weibull	Probit	Logistic
10^4	58	58	56	100	100
10^3	8.3	8.3	2.8	100	100
10^2	8.7×10^{-1}	8.7×10^{-1}	9.7×10^{-2}	1.66×10^{-6}	5.12×10^{-3}
10^1	8.7×10^{-2}	8.7×10^{-2}	3.32×10^{-3}	2.22×10^{-59}	1.46×10^{-11}
10^0	8.7×10^{-3}	8.7×10^{-3}	1.14×10^{-4}	0	4.16×10^{-20}
10^{-1}	8.7×10^{-4}	8.7×10^{-4}	3.89×10^{-6}	0	1.19×10^{-28}
10^{-2}	8.7×10^{-5}	8.7×10^{-5}	1.33×10^{-7}	0	3.38×10^{-37}
10^{-3}	8.7×10^{-6}	8.7×10^{-6}	4.55×10^{-9}	0	9.63×10^{-46}

Table X. Projections of Inhalation Risk to Humans for Exponential and beta-Poisson Models.

Data Source and Model	Infectious Dose 50% (95% interval % response)	Infectious Dose 1% (95% interval % response)
Muller [15]		
Exponential	11.7 (28% - 78%)	0.17 (0.5% - 2%)
beta-Poisson	11.5 (30% - 80%)	0.17 (0.5% - 4%)
Data Source and Model	**Lethal Dose 50% (95% interval % response)**	**Lethal Dose 1% (95% interval % response)**
Baskerville [10]		
Exponential	8×10^3 (21% – 89%)	115 (0.33% – 3.1%)
beta-Poisson	8×10^3 (23% – 85%)	115 (0.30% - 2.7%)

A dose of 0.17 CFU means 17 persons in 100 would receive on average one 1 CFU.

Table XI. LD QMRA Uncertainty Analysis

Project Phase & Factors	Extent of Uncertainty and Discussion
Dose-Response (DR) Modeling	
1. Animal model selection	Different animal models show several orders of magnitude difference in LD sensitivity. The guinea pig (GP) model is the most sensitive, and thus is a conservative choice (that is, is least likely to under-estimate human risk). The QMRA validation results suggest the GP model was an appropriate choice since predicted risks compared well to reported risks. Use of less sensitive animal data could have given several orders of magnitude under prediction of human risk.
2. DR data set selection	The comparison of multiple GP data sets from lethality studies shows a broad range, probably due to differences in Legionella strain virulence expression. Table VII compares the projections from the multiple GP data sets. The data demonstrating the most virulence were selected as a conservative choice. Most other data were within a factor of 2, with one higher set. The QMRA validation results suggest the data set selections were appropriate.
3. DR model selection	For several models, the low-dose extrapolations diverged from exposure probability below the experimental range. The QMRA validation results suggest the **β**-Poisson and exponential models were the appropriate choices. The probit and logistic results diverged the most, and if used, could have under-predicted risks by

	several orders of magnitude or predicted no risk whatsoever at low dose.
4. Dose scaling, inter-species adjustments	Adjustments for these are often applied in chemical risk assessment. Our QMRA findings suggest the mechanistic arguments against dose scaling are valid. Given the GP model selection basis, and the QMRA results, interspecies adjustments also appear unnecessary. The typical order of magnitude (10 x) factor for animal to human extrapolation commonly used in chemical risk assessment practice would have inflated the human LD risks inappropriately.
LD Outbreak Exposure Assessment – Whirlpool Spa*	
1. Water concentration estimates	No measurements were available in outbreak reports. The value used came from other whirlpool spa outbreak investigations that lacked other detail for exposure estimation. This value used is in a credible range but could be in error by an order of magnitude. A ten-fold change equates to a ten-fold change in exposure estimates and in risk estimates.
2. Air concentration estimates	The building ventilation rate used is from design guides since this was not available from reports. Other parameters came from investigation reports. Comparison to other reports Legionella in air near contaminated sources (see section 3.5) suggest the results are of a reasonable order of magnitude. A ten-fold change equates to a ten-fold change in risk estimates.
3. Exposure zones, distances	The zone information came from investigation reports and is not a source of uncertainty, except for time

	and distance patterns within a zone, which are not known. The sensitivity analyses [3] suggest this is not a lead source of variability.
4. Time in exposure zone	The work hours were reported as a mean and standard deviation for all workers. This parameter was a source of variability, but is not a significant contributor to uncertainty.
5. Inhalation rate	The inhalation rate distribution [2, 3, 85] was a major contributor to dose model variability but not to uncertainty.
LD Outbreak Exposure Assessment – Hot Springs Spas*	
1. Water content of Legionella, CFU/L	Outbreak investigation reports provided these data, which were obtained toward the end of the outbreak exposure period. There are no data showing if or how the water content changed over time. The actual concentration may have varied by an order of magnitude, which would alter predicted exposures and calculated LD rates similarly.
2. Water-to-air partitioning coefficient	This is a significant uncertainty. The partitioning coefficient used derived from endotoxins in air and water in a swimming pool environment. An order of magnitude error would contribute an order of magnitude error in the resulting exposure and risk estimates. This parameter nevertheless led to risk estimates that aligned well with reported LD rates.
3. Time exposed	This is a significant source of uncertainty. Actual case time exposed was not available, and we assumed a single visit for all, with a typical duration. The duration is unlikely to be an order of magnitude higher or

	lower, but multiple spa visits are possible for some of the spa users.
4. Inhalation rate	The inhalation rate distribution [2, 3, 85] was a major contributor to dose model variability but not to uncertainty.
LD QMRA Validation**	
1. Whirlpool spa reported disease rates	The rates are base on well-documented reports, with reliable data for both the numerator and denominator for the rate. These data do not contribute significantly to uncertainty.
2. Hot springs spa reported disease rates	The numerator (number of cases) for the reported risk may be more reliable than the denominator (number at risk via exposure). The number exposed is still arguably within a order of magnitude, with little likelihood of being higher, but potential for lower values, which would increase the reported rates of disease.
3. QMRA model predicted disease rates	Both the dose-response model extrapolation to humans and the estimated exposures for the outbreaks directly influence the uncertainty of the predicted risks. The uncertainties in the total LD QMRA model validation may rest more on the exposure portions than on the dose-response modeling. Further research to reduce the exposure uncertainties should be the most productive next stage for this LD QMRA model's development.

* for additional details, see Armstrong 2005, Armstrong and Haas 2007

** for additional details, see Armstrong 2005, Armstrong and Haas In Press

FIGURES

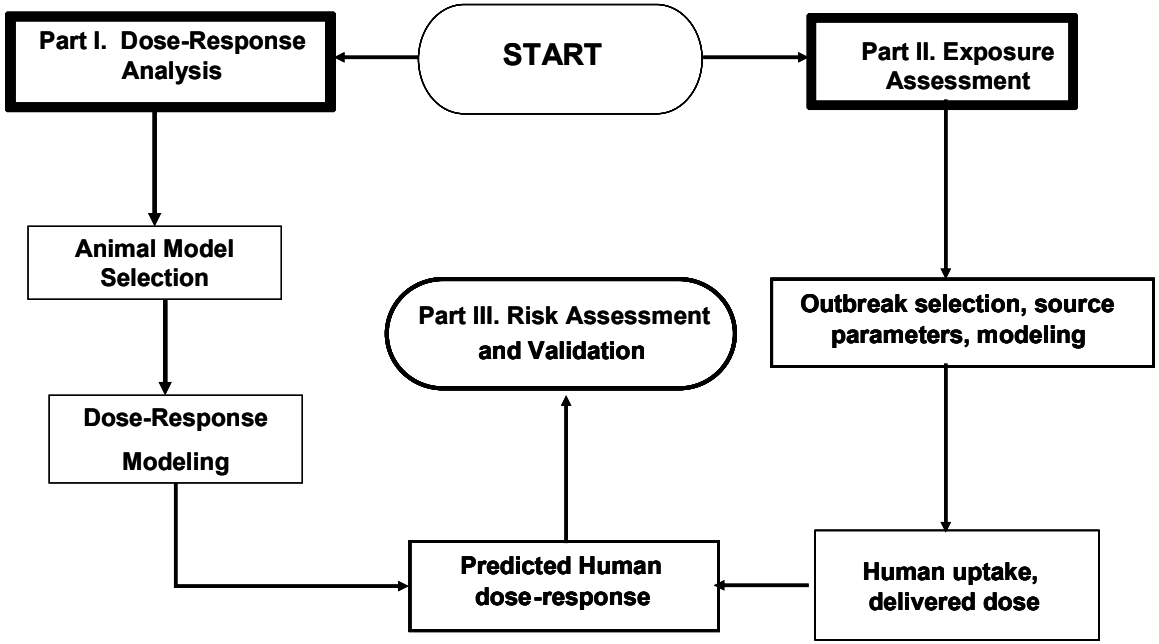

Figure 1. QMRA Plan. Information from the animal model selection and dose-response assessment and the exposure assessment converges at the predicted human dose-response stage and flows to the risk assessment and validation stage.

Exponential	$P_1(d) = 1 - e^{(-r \cdot d)}$
Approx. beta-Poisson	$P_1(d) = 1 - \left(1 + \frac{d}{\beta}\right)^{-\alpha}$
Exact beta-Poisson	$P_1(d) = 1 - {}_1F_1(\alpha, \alpha + \beta, -d)$
Weibull	$P_1(d) = 1 - e(-q_1 d^{q_2})$
Logistic	$P_1(d) = \frac{1}{1 + \exp[q_1 - q_2 \ln(d)]}$
Probit	$P_1(d) = \Phi\left(\frac{1}{q_2} \ln \frac{d}{q_1}\right)$ $where\ \Phi(y) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{y} \exp\left(-\frac{x^2}{2}\right) dx$

And where: *P1(d*) = the predicted response at a given dose d
r = a parameter for the exponential model
β = the beta parameter of the beta-Poisson models
α = the alpha parameter of the beta-Poisson models
1F1 = the Kummer confluent hypergeometric function
q1, q2 = parameters of the Weibull, Logistic, or Probit models

Figure 2. Dose-Response Model Equation List

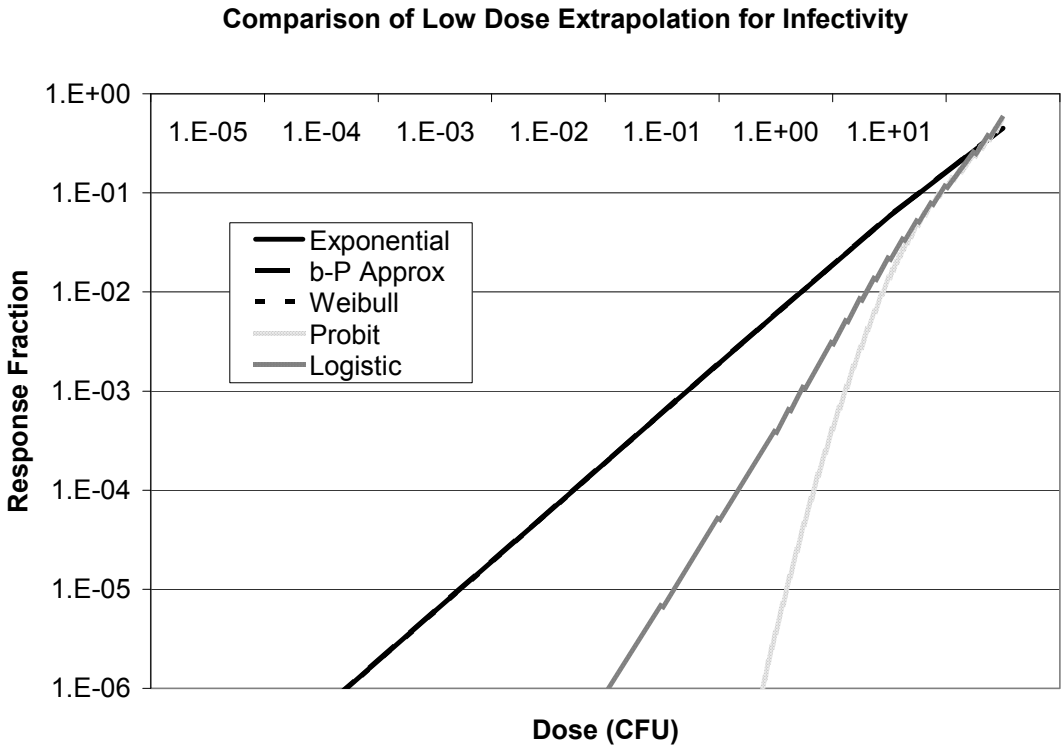

Figure 3. Comparison of the models' low dose extrapolation results for infection. Note that the Exponential, Approximate beta-Poisson and Weibull model results overlap, and are linear with dose. The probit and logistic models begin to fall below the dose probability. Based on the data of Muller et al. [15].

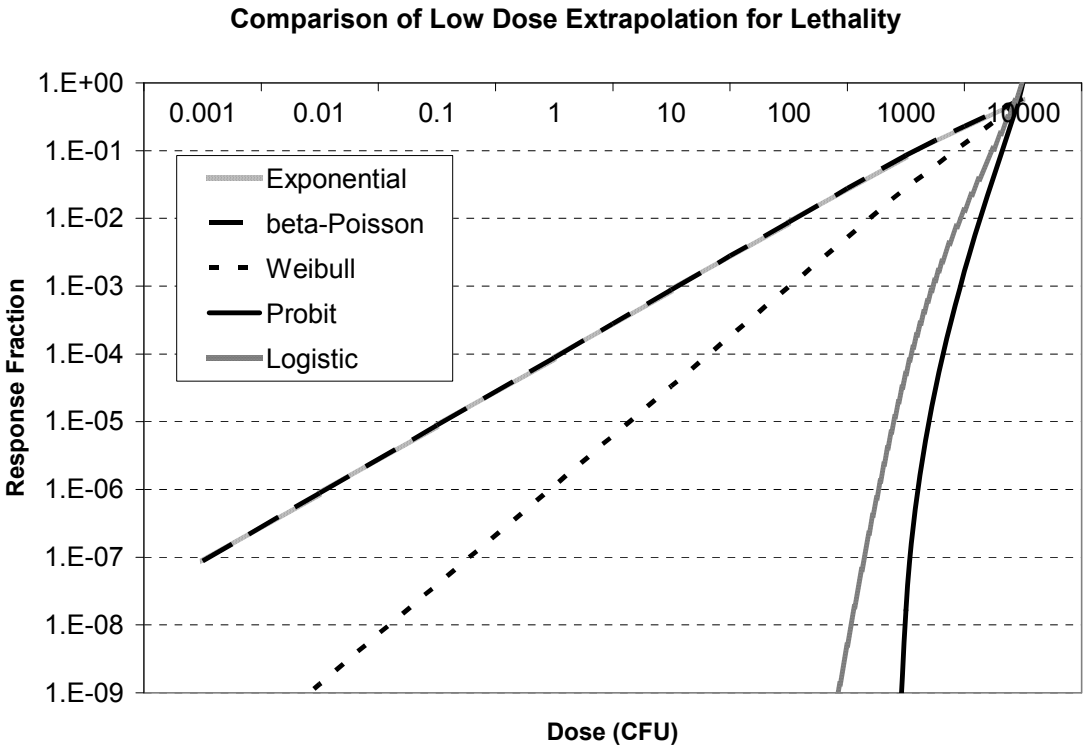

Figure 4. Comparison of the models' low dose extrapolation results for mortality. The exponential and beta-Poisson model results overlap. The logistic and probit model results rapidly diverge from the exposure probability and from the other models. Data from Baskerville et al. [10]