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Development of a dose–response model for *Naegleria fowleri*

Kara Dean, Mark H. Weir and Jade Mitchell

ABSTRACT

This study develops novel dose–response models for *Naegleria fowleri* from selected peer-reviewed experiments on the virulence based on the intranasal exposure pathway. One data set measured the response of mice intranasally inoculated with the amebae and the other study addressed the response of mice swimming in *N. fowleri* infected water. The measured response for both studies was death. All experimental data were best fit by the beta-Poisson dose–response model. The three swimming experiments could be pooled, and this is the final recommended model with an LD_{50} of 13,257 amebae. The results of this study provide a better estimate of the probability of the risk to *N. fowleri* exposure than the previous models developed based on an intravenous exposure.

An accurate dose–response model is the first step in quantifying the risk of free-living amebae like *N. fowleri*, which pose risks in recreational environments and have been detected in drinking water and premise plumbing systems. A better understanding of this risk will allow for risk management that limits the ability for pathogen growth, proliferation, and exposure.

Key words | dose–response, drinking water, *Naegleria fowleri*, recreational water, risk assessment

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INTRODUCTION

Free-living amebae (FLA) are present in freshwater sources. *Naegleria fowleri* is a thermophilic FLA commonly found in warm freshwater bodies and can survive temperatures of up to 40–45 °C (Kilvington & White 1985; Ma *et al.* 1990). *N. fowleri* is responsible for primary amebic meningoencephalitis (PAM), a highly fatal infection. The infection is acquired through forceful entry into the nasal canal such that the amebae enter the brain. This form of forceful inhalation into the nose could occur while swimming or diving in a body of water, or bath, or perhaps with the use of a neti-pot (Bright & Gerba 2017). Onset is rapid, with symptoms beginning with headache, fever, and nausea and quickly escalating to coma and seizures (Ma *et al.* 1990). PAM has a fatality rate of about 98%, affecting mostly children or young adults that have spent time swimming (CDC 2011; Bartrand *et al.* 2014). In a study conducted by the CDC, 121 cases of PAM were reviewed from 1937 to 2007. The majority of exposures occurred in warm, freshwater sources in the southern states of the USA (CDC 2008).

Although most incidences of PAM are seen in cases of swimming in warm waters, infections associated with bathing have also been reported (Ma *et al.* 1990; Blair *et al.* 2008). Some non-swimming cases reported were associated with contaminated premise plumbing systems or improper neti-pot usage (Bartrand *et al.* 2014). *N. fowleri* was present on both the third and fourth contaminant candidate list published by the Environmental Protection Agency because despite generally low outbreaks and occurrences, the health effects from infection are severe (Hoffman *et al.* 2009; U.S. EPA 2009, 2016). Concern over the colonization of Arizona wells by *N. fowleri* prompted a study where

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PCR detected *N. fowleri* DNA in 11 of the 143 tested wells (Blair *et al.* 2008).

In an effort to conserve water and pursue Green building designs, low flow conditions are becoming more common in water distribution systems. This is of particular concern with *N. fowleri* because it gains resistance when associated with biofilms. In a study conducted by Miller *et al.* (2015), *N. fowleri* established in attached biofilms were able to survive chlorine concentrations 30 times greater than the recommended amount (20 mg/L for 3 h and 10 mg/L for 48 h). The risk posed by *N. fowleri* to drinking water distribution systems may increase as the result of warmer temperatures from changing climate conditions and lower flows in plumbing systems. To better understand the risk posed by *N. fowleri* in these systems, quantitative microbial risk assessments (QMRA) are needed. QMRA is a widely used framework for risk characterization of waterborne pathogens in order to inform decisions about treatment, alternative design and selection (Haas *et al.* 2014).

A dose–response model to establish the mathematical relationship between exposure dose and risk for *N. fowleri* is needed for the forceful inhalation exposure route. This study aims to develop dose–response models from previously conducted studies on *N. fowleri* in the laboratory setting (John & Nussbaum 1983; John & Hoppe 1990). With these dose–response models, QMRAs can be performed to further inform the future design and treatment of drinking water distribution systems.

MATERIALS AND METHODS

Data

John & Hoppe (1990) determined patterns of susceptibility for small wild mammals exposed to *N. fowleri*. *N. fowleri* was instilled intranasally into a single naris of oppossums, raccoons, squirrels, muskrats, rabbits, mice, and rats using an Eppendorf pipette, and it was determined that mice were the most susceptible to infection. Male and female mice (ten in each group) were intranasally inoculated with the LEE strain of *N. fowleri* at doses ranging from 1,000 to 1,000,000 amebae per mouse. The data collected for male mice in this study are shown in Table 1 as Experiment 1.

John & Nussbaum (1983) studied the infection acquired by mice through swimming in amebae-contaminated water. Groups of ten CD1 mice were placed in a 1 L volume of distilled water containing different doses of amebae of the LEE strain of *N. fowleri* per mL of distilled water. Mice can normally float and keep their heads above water. To simulate an actual swimming exposure, groups of mice were put in the same container to create a crowded environment to spur swimming activity. After a specific time of swimming exposure (2.5, 5, 10, and 20 minutes), the mice were removed from the water and dried. The cumulative percentage of dead animals was recorded up to 28 days after exposure (John & Nussbaum 1983). The concentrations in the water for the 5, 10, and 20 minute studies were analyzed and are shown in Table 1 as Experiments 2, 3, and 4, respectively.

Analysis methods

The data were evaluated against specific quality criteria before modeling. This criterion consisted of ensuring that: (1) three or more graded doses were administered in the experiments; (2) at least three animals were tested in each dosing group; and (3) the data had a statistically significant trend by the Cochran–Armitage test. The studies evaluated included an adequate description of the dose administered, strain of the pathogen, host species, number of positive responses, and number of negative responses. Previously developed computer code in the statistical programming language, 'R' (www.r-project.org) (Weir *et al.* 2017) was used to fit the dose–response models using the method of maximum likelihood estimation, as described in Haas *et al.* (2014). Both the exponential dose–response model (Equation (1)) and the approximate form of the beta-Poisson dose–response model (Equation (2)) were fit to the data (Haas *et al.* 2014).

The exponential dose–response model is given by Equation (1) where $P(d)$ is the probability of response at dose, d, and the single parameter, k, is optimized during fitting and represents the probability that a single organism can survive to initiate the observed response.

$$P(d) = 1 - e^{-kd} \quad (1)$$

The approximate beta-Poisson model is given by Equation (2) where N_{50} is the median infective dose and α is a shape

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Table 1 | Dose–response data

Experiment	Pathogen	Exposure	Response	Dose[a] (no. of organisms)	Positive responses	Negative responses	Total responses	Resource
1	*N. fowleri*	Intranasal	Death	1,000	7	3	10	John & Hoppe (1990)
				10,000	8	2	10	
				100,000	10	0	10	
				1,000,000	10	0	10	
2	*N. fowleri*	Swimming for 5 min	Death	100	0	10	10	John & Nussbaum (1983)
				1,000	0	10	10	
				10,000	1	9	10	
				100,000	4	6	10	
				1,000,000	7	3	10	
3	*N. fowleri*	Swimming for 10 min	Death	100	0	10	10	John & Nussbaum (1983)
				1,000	1	9	10	
				10,000	4	6	10	
				100,000	6	4	10	
4	*N. fowleri*	Swimming for 20 min	Death	100	0	10	10	John & Nussbaum (1983)
				1,000	1	9	10	
				10,000	4	6	10	
				100,000	7	3	10	

[a]For Experiment 1, the dose shown is for number of amebae administered to each mouse. For Experiments 2–4, the dose shown is the concentration of amebae/mL of swimming water.

parameter (Haas *et al.* 1999; Teunis & Havelaar 2000). In this study, the N_{50} is actually an LD_{50}, the median lethal dose because the observed response in all data sets was death.

$$P(d) = 1 - \left[1 + \left(\frac{d}{N_{50}}\right) * (2^{1/\alpha} - 1)\right]^{-\alpha} \quad (2)$$

The 'rule of thumb' from Xie *et al.* (2017) was used to validate the application of the approximate beta-Poisson. These researchers propose $\Pr(0 < r < 1)|\hat{\alpha}, \hat{\beta}) > 0.99$ as a validity measure for the appropriate use of the approximate beta-Poisson with the constraint $\hat{\beta} > (22\,\hat{\alpha})^{0.50}$ for $0.02 < \hat{\alpha} < 2$. The full explanation for this approach is contained in Xie *et al.* (2017) and is therefore not reproduced here. This methodology was used to validate the use of the approximate beta-Poisson for the four independent data sets in this study. The results of this analysis are available in the Supplementary material as Table S1 (available with the online version of this paper).

To establish goodness of fit for the models, a comparison of the optimal value of the deviance to the critical χ^2 value at degrees of freedom equal to the number of doses minus the number of fitted parameters at an alpha value of 0.05 was conducted as previously described by Haas *et al.* (2014). In order to compare the fit of the two models for each data set, an assessment of the statistical significance of improvement of fit was made by comparing the reduction in minimized deviance with the critical χ^2 value at 1 degree of freedom between the two-parameter beta-Poisson model and the one-parameter exponential model. Confidence bands were estimated using a bootstrapping resampling technique.

When multiple data sets were available for the same pathogen, a statistical pooling analysis was performed to ascertain whether the data set had the same underlying distributions. A likelihood ratio test was used to determine if data could be pooled.

For the final model, the concentrations in Experiments 2, 3, and 4 had to be transformed into exposure doses. To do so, it was necessary to determine the quantity of water in milliliters that the mice could have possibly inhaled while swimming. None of the mice in the John & Nussbaum (1983) study died during the swimming event, so the assumed maximum volume of water and aerosols that the mice could have inhaled was less than the volume of water that would cause the mice to drown. To determine this quantity, the 3 milliliters used to drown rats in experimental drowning studies was multiplied by the size ratio of mouse and rat lungs, approximately 1:10 (Irvin & Bates 2003; Locali *et al.* 2006). The quantity of amebae that could be retained in the nasal passages, the target area of concern, was based off of

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the retention rate of 10 µm aerosols in the nasal passages of mice, the closest aerosol in size to the amebae of concern (Raabe *et al.* 1988). Once the concentrations were in exposure dose form, the modeling was performed once again as detailed above. Further details are available in the Supplementary material (available online).

RESULTS

Nasal cavity inoculation with *N. fowleri*

The beta-Poisson was the best fit model for the CD1 mice exposed intranasally to *N. fowleri* in Experiment 1. The minimized deviance of the exponential model exceeded the χ^2 value at degree of freedom one while the beta-Poisson model was well within the critical χ^2 value. Moreover, differences in deviances provided statistical significance of improvement of the beta-Poisson over the exponential model. The statistics of the two model fits to the animal studies are summarized in Table 2 and the best-fit model is shown in Figure 1. Due to the lack of intermediate responses, confidence bands could not reliably be estimated for the model. In Experiment 1, there were too few responses for dose groups where subjects exhibited a response less than 100%.

N. fowleri inhaled by swimming mice

The beta-Poisson model provided the best fit for the CD1 mice exposed to different concentrations in the water while swimming in Experiments 2, 3 and 4. The beta-Poisson models for each experiment are shown below.

Swimming for 5 minutes

The minimized deviance of the exponential model provided acceptable fit but difference in deviances (5.88) provided statistical significance of improvement of the beta-Poisson over the exponential model. The statistics of the two model fits to the animal study are summarized in Table 2 and the best-fit model with confidence bands is shown in Figure 2.

Swimming for 10 minutes

The beta-Poisson model was the best fit for the CD1 mice that swam for 10 minutes. The minimized deviance of the

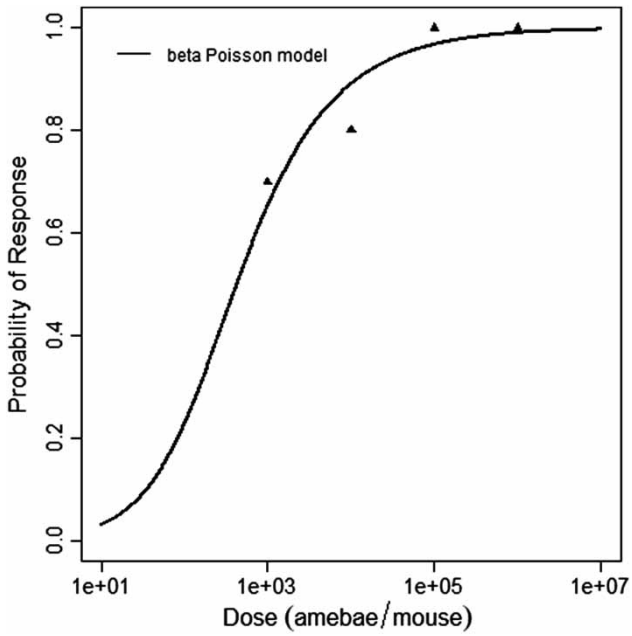

Figure 1 | Plot of beta-Poisson model for CD1 mice (male) exposed intranasally to *N. fowleri*.

Table 2 | Dose–response model statistics

Experiment	Model	Deviance	Δ	DF	$\chi^2_{\alpha,n-k}$	$\chi^2_{\alpha,1}$	Best fit	Parameters	LD_{50}
1	Exponential	11.28	9.64	3	7.81	3.84	Beta-Poisson	$\alpha = 0.536$; $\beta = 159.8$; $N_{50} = 422$	422
	Beta-Poisson	1.64		2	5.99				
2	Exponential	6.13	5.88	4	9.49	3.84	Beta-Poisson	$\alpha = 0.352$; $\beta = 32{,}132.2$; $N_{50} = 198{,}602$	198,602
	Beta-Poisson	0.25		3	7.81				
3	Exponential	7.89	7.55	3	7.81	3.84	Beta-Poisson	$\alpha = 0.241$; $\beta = 1{,}818.2$; $N_{50} = 30{,}447$	30,447
	Beta-Poisson	0.33		2	5.99				
4	Exponential	6.13	5.90	3	7.81	3.84	Beta-Poisson	$\alpha = 0.350$; $\beta = 3{,}162.8$; $N_{50} = 19{,}806$	19,806
	Beta-Poisson	0.23		2	5.99				

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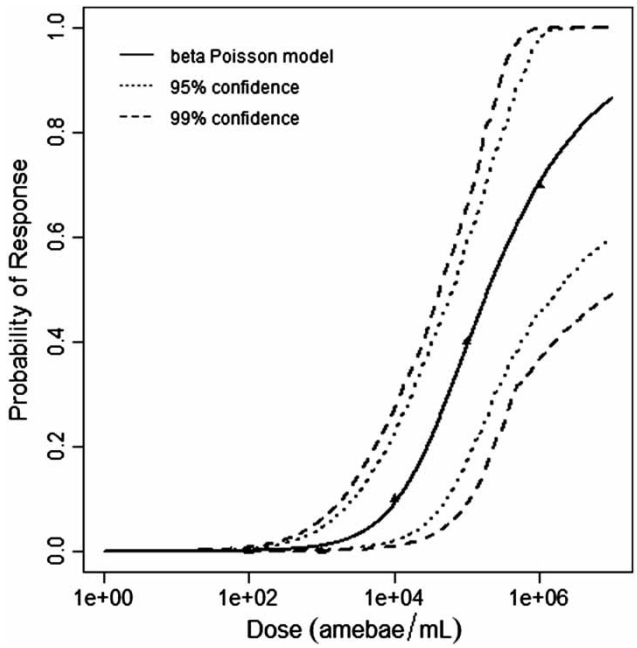

Figure 2 | Plot of beta-Poisson model for CD1 mice exposed via swimming for 5 minutes with upper and lower 95% and 99% confidence.

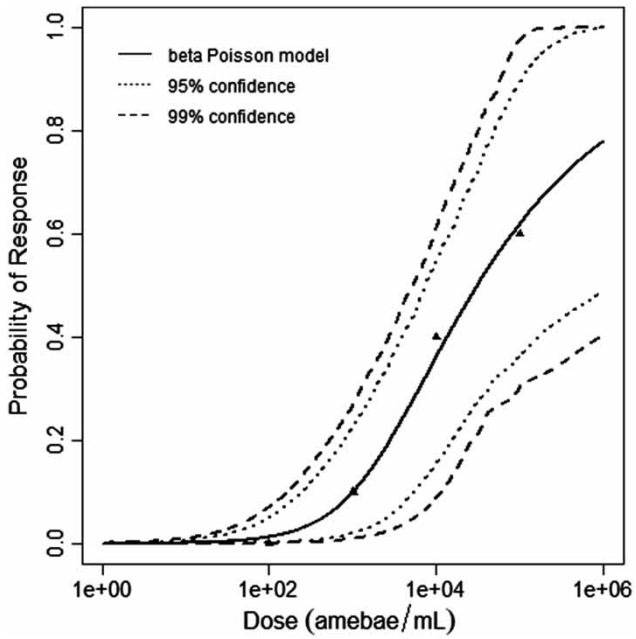

Figure 3 | Plot of beta-Poisson model for CD1 mice exposed via swimming for 10 minutes with upper and lower 95% and 99% confidence.

exponential model (7.88) exceeded the χ^2 value (7.81) at degree of freedom one and that of the beta-Poisson model was well within the critical value. Moreover, difference in deviances (7.55) provided statistical significance of improvement of the beta-Poisson over the exponential model. The statistics of the two model fits for Experiment 3 are summarized in Table 2 and the best-fit model with confidence bands is shown in Figure 3.

Swimming for 20 minutes

The minimized deviances of both the exponential as well as the beta-Poisson model provided acceptable fits. However, a difference in deviances (5.898) provided statistical significance of improvement of the beta-Poisson over the exponential model. The statistics of the two model fits are summarized in Table 2 and the best-fit model with confidence bands is shown in Figure 4.

Pooling analysis

Data of swimming episodes of CD1 mice for three different time periods (5 min, 10 min, and 20 min) in different concentrations of amebae per mL could be pooled. The value of difference in deviances between the sum of the individual best fits and pooled best fit was 6.584, which was less

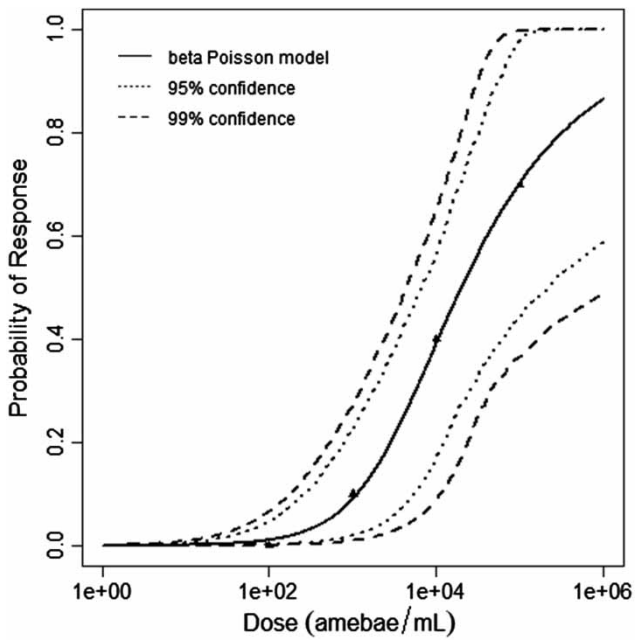

Figure 4 | Plot of beta-Poisson model for CD1 mice exposed via swimming for 20 minutes with upper and lower 95% and 99% confidence.

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Table 3 | Pooling statistics

Data set	Number of doses	Best fit model	Minimized deviance	DF	$\chi^2_{\alpha,n-k}$	$\chi^2_{\alpha,1}$	Parameters	LD_{50}
Pooling 2, 3, and 4 (Concentrations and doses*)	13	beta-Poisson	7.40	11	19.68	0.77	$\alpha = 0.226$ $N_{50} = 57{,}938$ $\alpha^* = 0.226$ $N50^* = 13{,}257$	57,983 13,257*

*Denotes the parameters for the model fit based on exposure dose values.

than the $\chi^2_{0.05,4}$ value (9.487). The summary and statistics of the pooling analysis are shown in Table 3 and Figure 5. The confidence of the bootstrapped parameters is shown in Figure 6. The beta-Poisson fit for the pooling analysis is the final recommended model.

Final recommended model

The pooling analysis revealed that all three swimming times could be represented by the same dose response model. Thus the concentrations in the water from Experiments 2, 3, and 4 were transformed into exposure doses. The average tidal volume for mice is 0.16 mL and as such a maximum of 2 breaths during the swimming event could have contained water or water aerosols (Fairchild 1972; Irvin & Bates 2003; Locali *et al.* 2006). Using this quantity and a retention rate in the nasal region of 71.5%, exposure doses were calculated (Raabe *et al.* 1988). For the more detailed calculations refer to the Supplementary material (available with the online version of this paper). The beta-Poisson was the best fitting model to the exposure dose data with a deviance of 7.40. The parameters are shown in Table 3, the model with 95% and 99% confidence is shown in Figure 7 and the confidence of the bootstrapped parameters is shown in Figure 8.

DISCUSSION

The studies by both John & Hoppe (1990) and John & Nussbaum (1983) evaluated intranasal exposure to the LEE strain

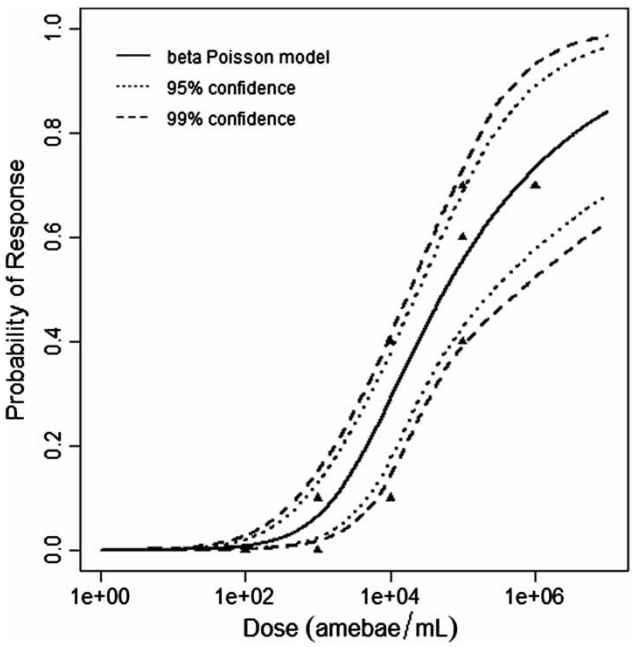

Figure 5 | Plot of beta-Poisson model for pooled data of CD1 mice swimming for 5, 10, and 20 minutes with upper and lower 95% and 99% confidence.

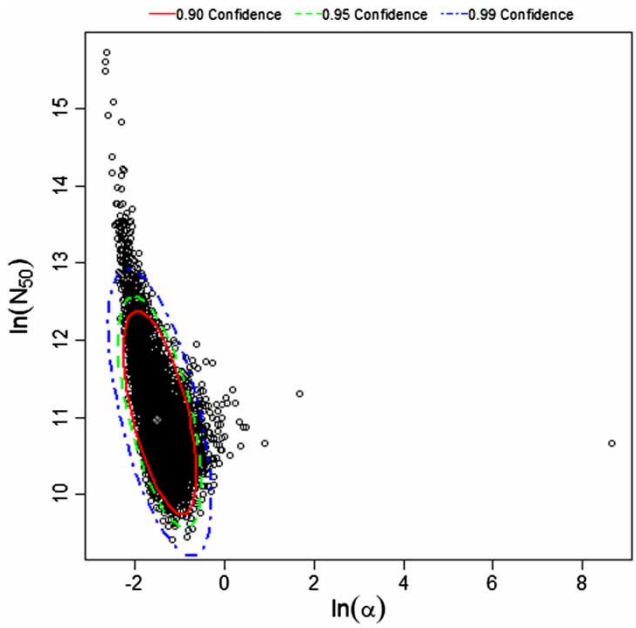

Figure 6 | Bootstrapped distribution of beta-Poisson parameter estimates for pooled data of CD1 mice swimming for 5, 10, and 20 minutes; the center marker (X) represents the maximum likelihood estimate.

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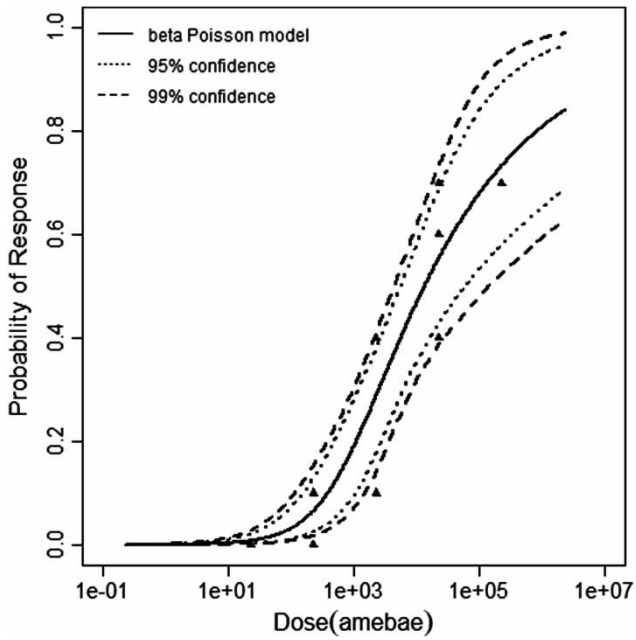

Figure 7 | Plot of beta-Poisson model for pooled data of CD1 mice exposed while swimming with upper and lower 95% and 99% confidence.

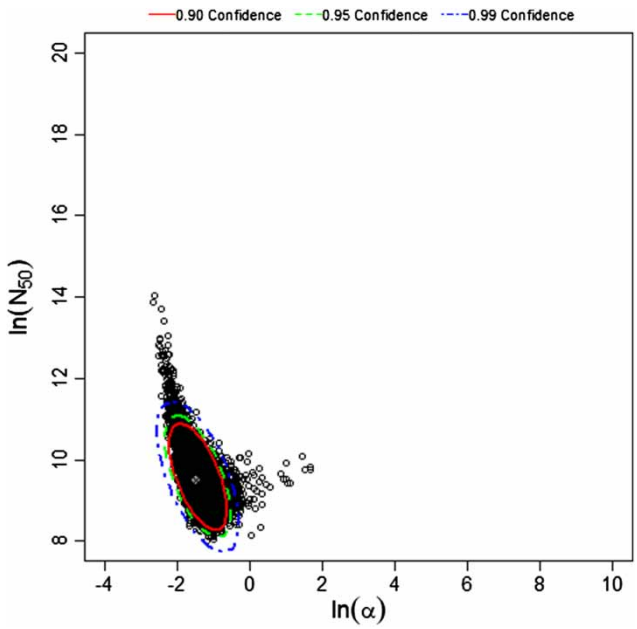

Figure 8 | Bootstrapped distribution of beta-Poisson parameter estimates for pooled data of CD1 mice exposed while swimming; the center marker (X) represents the maximum likelihood estimate.

of *N. fowleri* in CD1 mice. John & Hoppe (1990) inoculated intranasally into a single naris of immobilized animals using Eppendorf pipettes, while John & Nussbaum (1983) placed the animals into 1 L volumes of distilled water containing specified numbers of amebae to provide a tumultuous environment for swimming. The best fit model for the CD1 mice exposed intranasally via pipette and CD1 mice swimming at different time periods is the beta-Poisson, indicative of a heterogeneous response.

The beta-Poisson curve for the direct intranasal inoculation was steeper than the curves for the previously conducted dose–response study on intravenous exposure to *N. fowleri* (Huang 2013). This is indicative of a higher delivered dose reaching the receptive tissue, resulting in a higher probability of death. The LD_{50} for the intranasal inoculation was the lowest of the four experiments assessed in this study with a value of 422 amebae. This exposure route being more lethal is logical considering *N. fowleri* infection is associated with contaminated water being forced into the nasal cavity, an action that does not always naturally occur when just swimming. In Experiments 2–4, multiple mice were crowded into a single container to create a violent swimming environment where inhalation of water through the nose could occur. The individual model of different time periods shows the longer the swimming period, the lower the LD_{50} and the higher the probability of death for each dose. However, as the data sets of all the swimming periods could be pooled, this indicates that all the swimming episode cases can be described by a single model and would be considered mechanistically similar.

The pooling analysis revealed that all three swimming times could be represented by a singular dose response model. The concentrations were transformed into exposure doses assuming that the maximum possible volume of water was inhaled by the mice without causing them to drown. The pooled and final recommended model shown in Table 3 and Figure 7 had an LD_{50} of 13,257 amebae. This LD_{50} is over 30 times greater than the LD_{50} from the direct intranasal instillation exposure route. This disparity is logical considering the intranasal exposure deposited the amebae directly into the location of concern, making the migration of the amebae to the brain more likely. In a swimming event where amebae are inhaled with water and aerosols, the deposition into the mucous membrane is less likely.

The previously completed dose–response model for *N. fowleri* was completed with an intravenous exposure. The

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best-fit model for the intravenous exposure was the exponential model with an LD_{50} of 2,030,000 (Huang 2013). The model recommended in this study more accurately estimates the likelihood that *N. fowleri* amebae reaches the target receptor to initiate infection.

CONCLUSIONS

The dose–response models developed in this analysis are the first step in quantifying the risk *N. fowleri* poses to the population when present in drinking water distribution systems because they more closely match the expected exposure route. Although there are infrequent outbreaks and occurrences, the deadly effects of infection make *N. fowleri* a drinking water regulation focus (Hoffman *et al.* 2009). Although *N. fowleri* can be controlled with chemical and physical implementations in the premise plumbing system, it is the faltering or evading of these systems that causes concern. *N. fowleri* found in an area of a treated drinking water system with no detectable total chlorine residuals and temperatures greater than 30 °C was linked to a fatal infection in the United States (Cope *et al.* 2015). The recommended dose–response model developed in this study can be used to perform QMRAs to help risk managers protect human health and properly manage drinking water distribution systems to ensure safe drinking water.

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