Citation: Clin Transl Sci (2020) 13, 1060–1064; doi:10.1111/cts.12793

BRIEF REPORT

Quantification of Natural Growth of Two Strains of *Mycobacterium Marinum* for Translational Antituberculosis Drug Development

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The zebrafish infected with *Mycobacterium marinum* (*M. marinum*) is an attractive tuberculosis disease model, showing similar pathogenesis to *Mycobacterium tuberculosis* (*M. tuberculosis*) infections in humans. To translate pharmacological findings from this disease model to higher vertebrates, a quantitative understanding of the natural growth of *M. marinum* in comparison to the natural growth of *M. tuberculosis* is essential. Here, the natural growth of two strains of *M. marinum*, E11 and M^{USA}, is studied over an extended period using an established model-based approach, the multistate tuberculosis pharmacometric (MTP) model, for comparison to that of *M. tuberculosis*. Poikilotherm-derived strain E11 and human-derived strain M^{USA} were grown undisturbed up to 221 days and viability of cultures (colony forming unit (CFU)/mL) was determined by plating at different time points. Nonlinear mixed effects modeling using the MTP model quantified the bacterial growth, the transfer among fast, slow, and non-multiplying states, and the inoculi. Both strains showed initial logistic growth, reaching a maximum after 20–25 days for E11 and M^{USA}, respectively, followed by a decrease to a new plateau. Natural growth of both E11 and M^{USA} was best described with Gompertz growth functions. For E11, the inoculum was best described in the slow-multiplying state, for M^{USA} in the fast-multiplying state. Natural growth of E11 was most similar to that of *M. tuberculosis*, whereas M^{USA} showed more aggressive growth behavior. Characterization of natural growth of *M. marinum* and quantitative comparison with *M. tuberculosis* brings the zebrafish tuberculosis disease model closer to the quantitative translational pipeline of antituberculosis drug development.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Zebrafish infected with *Mycobacterium marinum* (*M. marinum*), a close relative to *Mycobacterium tuberculosis* (*M. tuberculosis*), show similar pathogenesis to *M. tuberculosis* infections in humans, making this a frequently used disease model to study tuberculosis pathology and antituberculosis pharmacology.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ The natural growth of two *M. marinum* strains (E11 and M^{USA}) is characterized over a period of > 200 days and quantitatively compared with the natural growth of *M. tuberculosis*.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ The poikilotherm-derived strain (E11) showed most similar growth parameters and behavior to those of *M. tuberculosis*. The human-derived strain (M^{USA}) showed more aggressive growth. This suggests that studies with E11 are more predictive of antibiotic effects on *M. tuberculosis*.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ Quantitative knowledge on the similarities and differences in natural growth between *M. marinum* strains and *M. tuberculosis* is essential for the translation of pharmacological findings on antituberculosis drugs between the zebrafish and higher vertebrates, including humans.

The zebrafish (*Danio rerio*) is an increasingly utilized disease model organism to study tuberculosis.[1,2] Infections of zebrafish embryos with *Mycobacterium marinum* (*M. marinum*), a close relative of *Mycobacterium tuberculosis* (*M. tuberculosis*),[3] show similar pathogenesis to *M. tuberculosis* infection in humans.[4] The zebrafish as a disease model organism has several

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Received: January 10, 2020; accepted: March 14, 2020. doi:10.1111/cts.12793

advantages. In contrast to experiments in higher vertebrates, like rodents, experiments with the zebrafish and specifically the zebrafish embryos and larvae are less time-consuming and resource-consuming. This is because of their high fecundity, small size enabling experiments in microtiter plates with high throughput potential, ease of noninvasive imaging due to its transparency, and limited ethical constraints.[5–7] As a whole vertebrate organism, it contains all relevant organ systems, including the immune response, which is an advantage over *in vitro* experiments. In tuberculosis research, this is especially relevant, as it has been shown that *M. tuberculosis* responds differently to treatment intracellularly inside macrophages, in comparison to standard (extracellular) *in vitro* experiments without macrophages.[8,9] Moreover, the interaction among pathogen, immune response, and treatment might be more relevant to study in the zebrafish, as it shows granuloma formation upon infection,[10] which is not observed in the mouse, the standard preclinical organism in tuberculosis research.[11] Translation of pharmacological findings from zebrafish embryos to higher vertebrates, including humans, requires a quantitative understanding of similarities and differences in pathophysiology between infecting mycobacteria. This forms the basis for a translational framework in drug development. One proposed model-based drug development approach for tuberculosis is the multistate tuberculosis pharmacometric (MTP) model.[12] This model distinguishes three states of multiplication for mycobacteria (i.e., fast, slow, and non-multiplying states) and characterizes growth rates and transfer rates among these states in the absence and in the presence of drugs, to better understand and translate drug effects. The MTP model has been successfully used in translation of pharmacological findings of *M. tuberculosis* treatment *in vitro*,[12] to mice[13] and humans.[14–18] To include the zebrafish in the translational pipeline for tuberculosis drug development, it is necessary to study the natural growth of *M. marinum* and quantitatively compare this to *M. tuberculosis*.

Here, the natural growth of *M. marinum* was studied over an extended period in two strains, one poikilotherm-derived (E11) and one human-derived (M^{USA}) strain. To facilitate a quantitative comparison to natural growth of *M. tuberculosis* and thereby assess the translational potential of pharmacological findings between species, the natural growth was characterized using the MTP model.[12]

METHODS

Total bacterial count assay

To replicate the experimental paradigm of the *M. tuberculosis* natural growth,[12] the two strains of *M. marinum* that are most commonly utilized in the zebrafish as a disease model are grown for an extended period of time in hypoxic conditions. To remain hypoxic, individual culture tubes can only be used for a single time point, as opening the tube for sampling introduces oxygen to the culture. *M. marinum* strains E11[19] and M^{USA20} were cultured in DifcoTM Middlebrook 7H9 broth medium (BD Biosciences, Franklin Lakes, NJ) containing 0.05% Tween80 (Merck KGaA, Darmstadt, Germany) and 10% BBLTM Middlebrook Albumin Dextrose Catalase Enrichment (BD Biosciences) at 30°C in closed tubes without disturbance up to 221 days. For each strain, three biological replicates from individually grown colonies were collected in individual tubes per time point, with a start inoculum at OD_{600} of 0.05, approximating 1×10^6 CFU/mL. At different time points (0, 8, 11, 18, 23, 28, 36, 42, 49, 56, 64, 69, 77, 85, 92, 112, 114, and 221 days), the viability of the cultures, defined as CFUs per mL, was determined by plating a series of 10-fold dilutions (ranging from 1:10 to 1:1,000,000) in triplicate on Difco Middlebrook 7H10 agar (BD Biosciences) containing 5% glycerol (Sigma-Aldrich, Saint Louis, MO) and 10% BBLTM Middlebrook Oleic Albumin Dextrose Catalase Enrichment (BD Biosciences).

Model-based quantification of natural growth

Nonlinear mixed effects modeling using the First Order Conditional Estimation was performed with NONMEM (version 7.3)[21] through interfaces Pirana (version 2.9.6) and PsN (version 4.7.0). Graphical outputs were generated with R (version 3.5.0) through interface Rstudio (version 1.1.383; RStudio, Boston, MA).

The previously developed MTP model[12] for *M. tuberculosis* was used to fit the CFU/mL of both *M. marinum* strains. The model structure consisted of three bacterial states for fast, slow, and non-multiplying bacteria, where the sum of fast and slow-multiplying bacteria was assumed to equal CFUs/mL:

$$\frac{dF}{dt} = k_g \cdot F + k_{SF} \cdot S - k_{FS} \cdot F - k_{FN} \cdot F$$

$$\frac{dS}{dt} = k_{FS} \cdot F + k_{NS} \cdot N - k_{SF} \cdot S - k_{SN} \cdot S$$

$$\frac{dN}{dt} = k_{FN} \cdot F + k_{SN} \cdot S - k_{NS} \cdot N$$

in which F represented the fast-multiplying, S the slow-multiplying, and N the non-multiplying state, k_g represents the growth function, and k-values represent transfer rates from the state first mentioned in the subscript to the state mentioned second. To ascertain mathematical identifiability, the transfer rates were fixed to values obtained in the MTP model for *M. tuberculosis*, with the exception of the transfer rate between the fast and slow-multiplying state, which was estimated. Without fixing these parameters, the MTP model was not identifiable and, therefore, the translational potential of this study would be lost. By fixing the transfer rates to those found in *M. tuberculosis*, it was implicitly assumed that the natural growth behavior as a result of the transfer between the different states was similar between *M. tuberculosis* and *M. marinum*. This was considered not biologically unreasonable, because both *M. tuberculosis* and *M. marinum* share a genetic program for (intracellular) growth[4] and genetic functions required for dormant infections are found in both genomes.[22]

To quantify the growth rate for the *M. marinum* strains, exponential, Gompertz, and logistic functions were tested, which assumed unrestricted (exponential) or restricted (Gompertz, logistic) growth based on the capacity of the system:

$$k_g = k_{g,e}$$

$$k_g = k_{g,G} \cdot \log\left(\frac{B_{max}}{F+S+N}\right)$$

$$k_g = k_{g,l} \cdot \log\left(B_{max} - (F+S+N)\right)$$

where $k_{g,e}$ is the exponential growth rate, $k_{g,G}$ is the Gompertz growth rate, B_{max} is the maximum capacity of the system, and $k_{g,l}$ is the logistic growth rate. The transfer rates between the fast and slow-multiplying state were tested as a constant, or with capacity-dependent or time-dependent functions. Capacity-dependent transfer was considered in case an excess total bacterium led to an increased transfer from the fast-multiplying to slow-multiplying state. For the time dependency, linear as well as sigmoidal and exponential functions were tested, as described previously.[12] The inoculum at $t = 0$ was tested to either be all in the fast-multiplying state (F_0), or all in the slow-multiplying state (S_0), or both F_0 and S_0 were estimated. This was based on the assumption that an *in vitro* infection starting from fresh cultures would consist of multiplying bacteria, either fast and/or slow. No bacteria were assumed to be non-multiplying at the logistic growth phase at the start of the experiment, therefore, N_0 was fixed to zero.

Two levels of variability were tested to distinguish biological from experimental variability. However, biological variability could not be estimated with acceptable precision. Residual error was quantified as a proportional error.

Model selection criteria were biological plausibility of parameter estimates, as well as standard goodness-of-fit plots.[23] The likelihood ratio test was used to test statistical significance between nested models, where a drop in objective function value (ΔOFV) of 3.84 corresponded to $P = 0.05$ with a single degree of freedom, assuming the χ^2-distribution. Non-nested models were compared by Akaike Information Criterion (AIC), which penalizes ΔOFV with the number of additional parameters.[24] Model diagnostics were performed by goodness-of-fit plots and a visual predictive check.[23]

RESULTS

Total bacterial count M. marinum

Both *M. marinum* strains show an initial logistic growth phase as represented by the steep increase in CFU/mL in **Figure 1a,b**. This growth phase reaches a maximum for E11 at 1×10^9 CFU/mL after 20 days, after which it decreases to a plateau of 1×10^7 CFU/mL after 100 days. For M^{USA}, an initial maximum was reached after 25 days at 3×10^8 CFU/mL. Its decrease to a plateau similar to E11 was, however, less clear, due to high variability.

MTP model predictions of natural growth

For strain E11, the MTP model with a Gompertz growth function, constant transfer rates between the fast and slow-multiplying states, and the inoculum estimated to be exclusively in the slow-multiplying state, resulted in the best fit. Both a linear and logistic growth function decreased the fit substantially (ΔAIC = 40.8 and 39.2, respectively). Estimating the inoculum in the fast state (F_0) was statistically significantly worse (dOFV = 8.51; $P < 0.005$).

For strain M^{USA}, the MTP model with Gompertz growth function, constant transfer rates between the fast-multiplying and slow-multiplying states, and the inoculum estimated to be exclusively in the fast-multiplying state resulted in the best fit. Both a linear and logistic growth function decreased the fit substantially (ΔAIC = 23.3 and 34.4, respectively). Model fit was very similar when parameterizing transfer between fast and slow states as capacity-dependent or time-dependent, but based on precision of parameter estimates and parsimony, a constant transfer rate that requires less parameters, was selected for the final model. All tested approaches for the inoculum resulted in similar fits, based on parameter precision an inoculum in F_0 was selected.

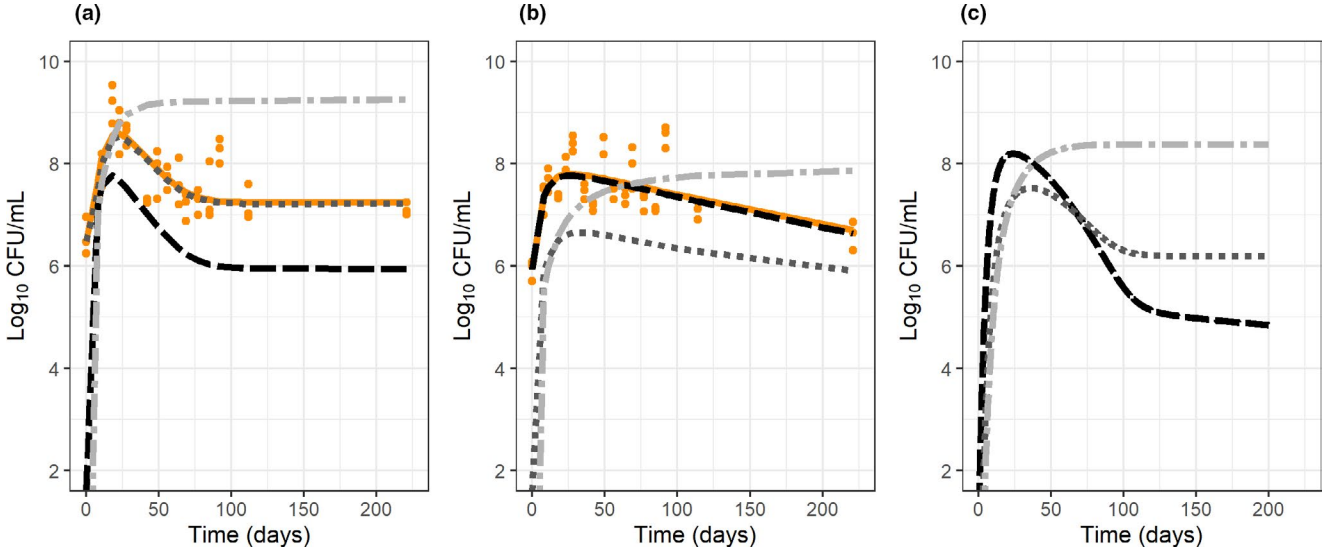

Figure 1 Natural growth of *Mycobacterium marinum* strains E11 (**a**) and M^{USA} (**b**) for hypoxic growth during 221 days. Previously published natural growth for *Mycobacterium tuberculosis*[12] (**c**) is included for comparison. Symbols: observed CFU/mL for the *M. marinum* strains. Lines: prediction of bacterial counts in fast-multiplying (black dashed line), slow-multiplying (dark grey dotted line), and non-multiplying (light grey dot-dashed line) states. The sum of fast-multiplying and slow-multiplying states (orange solid line) is assumed to equal colony forming unit (CFU)/mL.

Table 1 Parameter estimates obtained with the final MTP model for natural growth of *Mycobacterium marinum* strain E11 and M^{USA} upon undisturbed, hypoxic growth for 221 days

	M. marinum E11		*M. marinum* M^{USA}		*M. tuberculosis*[12]
Parameter	Estimate	Relative standard error (%)	Estimate	Relative standard error (%)	Estimate
k_G (day^{-1})	0.188	4.1	0.169	19.3	0.206
k_{FS} (day^{-1})	1.33[a]	25	0.0157[a]	9.5	0.00166[a]
B_{max} (mL^{-1})	4.99×10^{11}	160	7.91×10^{7}	7.7	242×10^{6}
F_0 (mL^{-1})	0 FIX		8.51×10^{5}	21.2	4.1
S_0 (mL^{-1})	3.07×10^{6}	40.1	0 FIX		9,770
k_{FN} (day^{-1})	8.97×10^{-7} FIX		8.97×10^{-7} FIX		8.97×10^{-7}
k_{SF} (day^{-1})	0.0145 FIX		0.0145 FIX		0.0145
k_{SN} (day^{-1})	0.186 FIX		0.186 FIX		0.186
k_{NS} (day^{-1})	0.00123 FIX		0.00123 FIX		0.00123
Variance of proportional residual error	0.227	9.5	0.205	9.5	0.407

Parameters reported for *Mycobacterium tuberculosis* with this model[12] are depicted for comparison.
[a]B_{max}, maximum capacity of the system; F_0, inoculum in the fast-multiplying state; k_g, growth function, remaining *k*-values represent transfer rates from the state first mentioned in the subscript to the state mentioned second; MTP, multistate tuberculosis pharmacometric; S_0, inoculum in the slow-multiplying state.
[a]*M. marinum*, transfer between fast-multiplying and slow-multiplying state was a constant function, for *M. tuberculosis*, transfer between fast-multiplying and slow-multiplying state was a time-dependent linear function ($k_{FS} = k_{FSlin} \cdot time$)[12] with unit day^{-2}.

Final model predictions for number of bacteria in all three states as well as observed CFU/mL are shown in **Figure 1a,b**, final parameter estimates are given in **Table 1**, diagnostic plots are given in **Supplementary Figures –S1–S4**. Model prediction of the three states for strain E11 shows relatively quick transfer from F to S and N, whereas for strain M^{USA} the F state is the major contributor to the total bacterial count. The growth rate constant of E11 and M^{USA}, 0.188 and 0.169 day^{-1}, respectively, was comparable to that of *M. tuberculosis* (0.206 day^{-1}).[12] For *M. tuberculosis*, S_0 was reported to contain the majority of the total inoculum,[12] which was similar to E11 but not M^{USA}.

DISCUSSION

To utilize *M. marinum* in the zebrafish tuberculosis disease model, quantification of its natural growth over an extended period is essential to study similarities and differences with *M. tuberculosis.* We made a quantitative comparison between *M. marinum* strains and with *M. tuberculosis* using the established MTP model. This modeling and simulation approach is able to use the gained knowledge on potential differences when translating effects between species by integrating the quantified natural growth with the drug effects on the different states and translational factors correcting for between-species differences.[15] A more parsimonious model with less compartments or parameters would result in similar predictions of CFU/mL for *M. marinum*, but would as a mere descriptive model lose its translational strength. The quantification of the natural growth of *M. marinum* in absence of therapeutic agents is the first step in the translational framework of the zebrafish as disease model for tuberculosis. A next step is to include drug effects on the different states of *M. marinum* in the model, similar to those already quantified on the different states of *M. tuberculosis*, with which it can be compared. A third step is the infection and treatment of zebrafish with *M. marinum* to quantify the drug effect within the context of a whole vertebrate, including an innate immune response. It is the advantage of the zebrafish as disease model with high-throughput potential to screen many different compounds, in contrast to infection experiments in higher vertebrates.[25] Utilizing the MTP-based quantification of the *M.* marinum infection and treatment in the zebrafish will enable translation of the observations in this new model organism to higher vertebrates, including humans,[15,16] an essential step in antituberculosis drug development.

Comparison of *M. marinum* to *M. tuberculosis* shows similar growth rates, especially for strain E11, which also shows most similar model prediction for the bacterial counts in the three states (**Figure c1**), suggesting E11 to be preferable when studying tuberculosis pathology in zebrafish. The poikilotherm-derived E11 showed, with the prominence of the slow-multiplying state, a more latent growth behavior than the human-derived M^{USA}, which remained fast-multiplying for an extended period, suggesting more aggressive growth behavior. These characteristics are consistent with literature.[19]

In conclusion, the natural growth of *M. marinum* was characterized and a quantitative comparison to *M. tuberculosis* was made using an established model-based approach. The improved understanding of the mycobacterial pathogen of the zebrafish tuberculosis disease model brings this promising and versatile model organism one step closer to the quantitative translational pipeline of antituberculosis drug development.

Supporting Information. Supplementary information accompanies this paper on the *Clinical and Translational Science* website (www.cts-journal.com).

Funding. The research leading to these results has received funding from the Innovative Medicines Initiative Joint Undertaking (www.imi.europe.eu) under grant agreement no 115337, resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2007–2013) and EFPIA companies' in kind contribution.

Conflict of Interest. The authors declared no conflict of interest.

Author Contributions. R.C.vW., A.M.vdS., E.H.J.K., H.P.S., U.S.H.S., and P.H.vdG. wrote the manuscript. R.C.vW., A.M.vdS., E.H.J.K., H.P.S., U.S.H.S., and P.H.vdG. designed the research. A.M.vdS. and T.V. performed the research. R.C.vW. and E.H.J.K. analyzed the data.

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