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Optimization and validation of *Mycobacterium marinum*-induced adult zebrafish model for evaluation of oral anti-tuberculosis drugs

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ABSTRACT

Introduction: *Mycobacterium marinum* has emerged as a suitable species for induction of tuberculosis-like disease in zebrafish, and various zebrafish models (larval and adult) for drug screening have been proposed in the literature. It is believed that an adult zebrafish model is more useful in drug screening because, apart from assessment of efficacy, one can obtain data on dosage, pharmacokinetics and overall health improvement. This study suggests a simple, cost-effective and resource-efficient protocol for screening of anti-tuberculosis drugs.

Methods: The parameters used for assessment of infection as well as anti-bacterial response were: (a) bacterial count; and (b) body weight change. An optimization study was conducted to establish the concentration of bacteria required to produce a reproducible phenotype of tuberculosis (TB). A negative control (Amoxicillin) and anti-mycobacterial drugs (Isoniazid, Rifampicin, Moxifloxacin, Ethambutol and Isoniazid + Rifampicin) were used for validation of the protocol. All the drugs were administered orally.

Results: An intra-peritoneal inoculation of 0.75 million bacteria/fish was optimized for the model. All the anti-tuberculosis drugs showed efficacy in this model, whereas the negative control did not show any signs of reversing the parameters of *M. marinum* infection.

Discussion: Adult zebrafish model of *M. marinum*-induced tuberculosis has not been fully exploited as a drug screening tool. In the present report, a protocol is suggested that is simple, reproducible and resource-efficient for screening of anti-tuberculosis agents. This protocol is an attempt to refine the published protocols and use this model as a surrogate model of human TB for the purpose of drug screening.

Introduction

Human tuberculosis (TB), caused by Myocbacterium tuberculosis, is one of the major health challenges faced by the developing and underdeveloped countries across the world. Many academic as well as industrial researchers are engaged in understanding this disease, as well as finding a therapeutic cure for this disease using animal experimentation. The most

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commonly used laboratory animals, including mouse, guinea pig and rabbit models, have limitations in terms of representation of the disease process in human TB [1,2]. Nonhuman primates are considered to be the most predictive models to mimic human TB [3], but their use has been limited because of cost and ethical issues. Therefore, there is a need to have alternate models for the study of TB, as well as to screen potential anti-TB agents during drug discovery.

Mycobacterium marinum is a marine counterpart of *M. tuberculosis* [4] and performs all essential functions required to elicit a granulomatous disease [5,6]. It grows optimally at temperatures between 33 °C and 35 °C and can thus produce TB in ectotherms–zebrafish being the most popular amongst them. Furthermore, the innate and adaptive immune system of zebrafish is similar to that of the human system [7]. *M. marinum* can be safely handled using BSL-II precautions, because its infection in humans is quite localized and does not impact the user systemically unless the host is severely immunecompromised [8].

This current study is based on the previous literature, to use zebrafish as a surrogate model for drug screening, as a step between *in vitro* assessment and *in vivo* pharmacological evaluation in mammalian models. A simple adult zebrafish model was standardized to screen compounds using a protocol that is resource efficient, inexpensive, involves simple techniques and does not require sophisticated instrumentation. This protocol is based on parameters of: (a) bacterial count, and (b) body weight change. Furthermore, oral drug administration was employed to ensure the systemic delivery of drugs and ascertain the dose of the drugs administered to each fish. The protocol has been validated using standard drugs wherein isoniazid, rifampicin, moxifloxacin, ethambutol and isoniazid + rifampicin (combination) were used as positive controls, whereas, amoxicillin was used as a negative control.

Animal ethics statement

Experiments were performed following animal ethics guidelines of the institutions and were performed under the supervision of a licensed veterinarian. The mortality rate due to infection in adult zebrafish was similar to that observed in other mammalian models of TB [see Results Section 4.1].

Methods

Zebrafish maintenance

Zebrafish were maintained as per Guidelines for Use of Zebrafish in the NIH Intramural Research Program [9] and the Zebrafish Book [10]. Zebrafish were obtained from Vikrant Aquaculture, Mumbai, India, and were maintained at BITS-Pilani, Hyderabad campus, India as per the procedures mentioned earlier [11,12]. Briefly, all the fish were taken care to acclimatize for a week at 26–28 °C and at conditions of 14:10 hr. (light:dark) every day. The fish were allowed to swim in separate chambers filled with filtered water containing 0.2% sea salt and were fed with dry food (procured from the same vendor) at three regular intervals daily. Fish were observed to be healthy through their feeding and swimming activities and with a weight range of 500 mg. The healthy fish were further selected to conduct the study.

M. marinum strains, culture condition and inoculation

M. marinum strains used for this study were derived from a human clinical isolate, strain M (ATCC BAA-535), and were grown at 30 °C in Middlebrook 7H9 broth (HiMedia) supplemented with Middlebrook OADC Growth supplement (HiMedia) and 0.05% Tween 80 or on Middlebrook 7H10agar (HiMedia) supplemented with Middlebrook OADC Growth supplement (HiMedia). Infected fish homogenates were plated in 48 well plates using Middlebrook 7H9 broth (HiMedia) supplemented with amphotericin B (10 mg/liter) and polymyxin B (20 mg/liter), to avoid contamination with normal flora. Cultures used in infections were grown to an optical density at 600 nm of 1.0 and maintained at −80 °C in 1-ml aliquots with 10% glycerol [2]. Intraperitoneal administration (i.p.) was used for bacterial inoculation, wherein a maximum volume of 15 μl/fish was injected using a 29-gauge insulin syringe to avoid injury-induced stress based on methods described in the literature [12,13].

Drug, vehicle and drug administration

The standard drugs Isoniazid, Rifampicin, Ethambutol, Moxifloxacin, and Amoxicillin were procured from Sigma Aldrich, and Tween 80 was procured from NICE laboratories. All other routine chemicals were procured locally. All drugs were administered orally using a recently reported method [11,14]. This method allows the calculation of the oral dose of the drugs in terms of milligrams per kilograms (mg/kg), which is very useful in ascertaining the vital parameter of drug dosage required for ranking of molecules and taking decisions in a screening program. The authors that proposed this method have demonstrated the credibility of the method by substantiated pharmacokinetics and pharmacology data.

Optimization study

A study was conducted to optimize the concentration of bacteria needed to produce a reproducible phenotype of zebrafish TB. Healthy fish were grouped into four groups ($n = 15$/group) and *M. marinum* cultures were injected into the fish (inside a BSL-II hood) at inoculums of 0.5 (Group I), 0.6 (Group II) and 0.75 (Group III) million bacteria respectively. Two time points viz. day 7 and 14 were used to sacrifice the fish and results were determined using Most Probable Number (MPN) assay ($n = 6$) and body weight ($n = 10$). Before sacrificing, the fish were allowed to swim in 1.5 mg/ml of Kanamycin Sulfate for 45 min at 27 °C, to prevent any cross-infection [2]. Thereafter, the fish were homogenized and processed for MPN assay as per a published protocol [15]. MPN values were finally calculated using standard statistical methods [16]. The survival probability curve of Group III fish was also plotted by conducting a separate experiment on 90 fish based on Kaplan–Meier survival analysis [17].

Validation study

An *in vivo* protocol using the standard drugs was further designed. It was performed as a two-week study: infection stage (0–7th day) and treatment stage (8th–14th day). Eighty fish were inoculated with an optimized count of *M. marinum*. A dose optimization study resulted in finalizing the following drug doses: amoxicillin (10 mg/kg), isoniazid (10 mg/kg), rifampicin (5 mg/kg), moxifloxacin (5 mg/kg), ethambutol (10 mg/kg) and a combination of isoniazid (5 mg/kg) + rifampicin (2.5 mg/kg). The drug solutions (diluted with vehicle Tween 80) were prepared considering the average body weights of the fish in each group. 5 μl of each drug ($n = 10$ in each group) was administered orally. This dosing was done for a week (8th–14th day), and the results were determined using MPN assay ($n = 6$) and body weight ($n = 10$).

Results

Optimization study

The lesions observed in infected fish were redness with squamous eruptions (in the form of white fibers) (Fig. 1).

The MPN assay showed a clear dose response in the increase of the bacterial counts (Fig. 2). Group I showed approximately 1.2- and 1.8-fold increase in MPN on days 7 and 14 respectively. Group II showed an increase of 1.5- and 3.1-fold increase in bacterial counts on the days of sacrifice (days 7 and 14). The highest increase was seen in Group III with an increase of 4.1 on day 7 post infections and a huge 12.8-fold increase on day 14 post infection.

Body weight reduction was seen in a dose response manner (Fig. 3). The mean body weight reduction in the control group was 1.7 ± 2% and 0 ± 1.8% on days 8 and 14 post infection, respectively. Whereas, in the infected groups, the reduction was significantly higher within 8 days post infection and was observed to be 18.5 ± 1.5%, 29.5 ± 1.8% and 31.7 ± 1.4% in Groups I, II and III, respectively. On day 14, there was a further reduction in body weights in all three groups by 22.3 ± 1.6%, 33.2 ± 1.8% and 41.2 ± 1.1% in Groups I, II and III, respectively.

In order to ensure that Group III was suitable for conduct of screening experiments, a survival probability assessment (Fig. 4) was conducted on 90 fish after inoculation of 0.75 million bacteria and observation for 14 days for survival. At the end of 14 days, a survival probability of 0.71 suggested this dose to be robust and suitable for conducting screening experiments with a substantial sample size surviving for measuring the response of test drugs. It is reported that almost half (47%) of the experiments conducted on murine models of TB are based on the criterion of lethality [18], and the biological significance for efficacy in such models is generally considered to be a 20% improvement in survival. Therefore, the survival probability of >0.7 in this model ought to be sufficiently "humane" for the purpose of drug efficacy evaluation.

Therefore, Group III was the group that showed substantial symptoms and a high lesion score, >10-fold increase in MPN, >40% reduction in body weight, and >0.7 survivability. Thus, an intra-peritoneal inoculation of 0.75 million bacteria/fish was selected as the suitable paradigm for *M. marinum* infection model in adult zebrafish.

Validation study

The dose optimization study helped in selecting the doses for the final study. Based on the visual observations the dose groups selected for the final study were: Amoxicillin (10 mg/kg), Isoniazid (10 mg/kg), Rifampicin (5 mg/kg), Moxifloxacin (5 mg/kg), Ethambutol (10 mg/kg) and a combination of Isoniazid (5 mg/kg) + Rifampicin (2.5 mg/kg).

All the parameters, i.e., MPN assay results (Fig. 5) and body weight reduction observations (Fig. 6b) demonstrated that the anti-tuberculosis drugs were efficacious in this model whereas the negative control Amoxicillin did not show any signs of efficacy in reversing the parameters of *M. marinum* infection.

The average bacterial counts were >12-fold higher in the infected group and the data was consistent with the results seen in the optimization study. The Amoxicillin treated group also showed MPN equivalent to the infection control fish. Fish treated with all the drugs showed almost complete

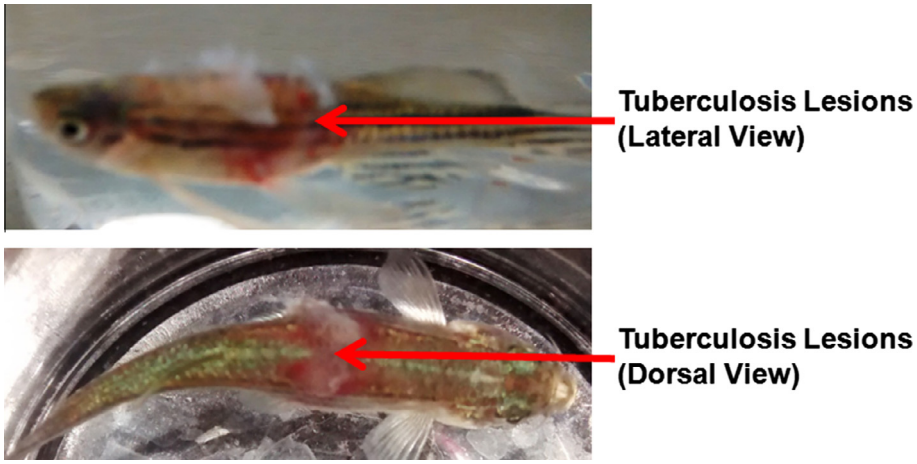

Fig. 1 – *M. marinum*-induced adult zebrafish with red lesions & squamous eruptions on dorsal and lateral sides observed during the study.

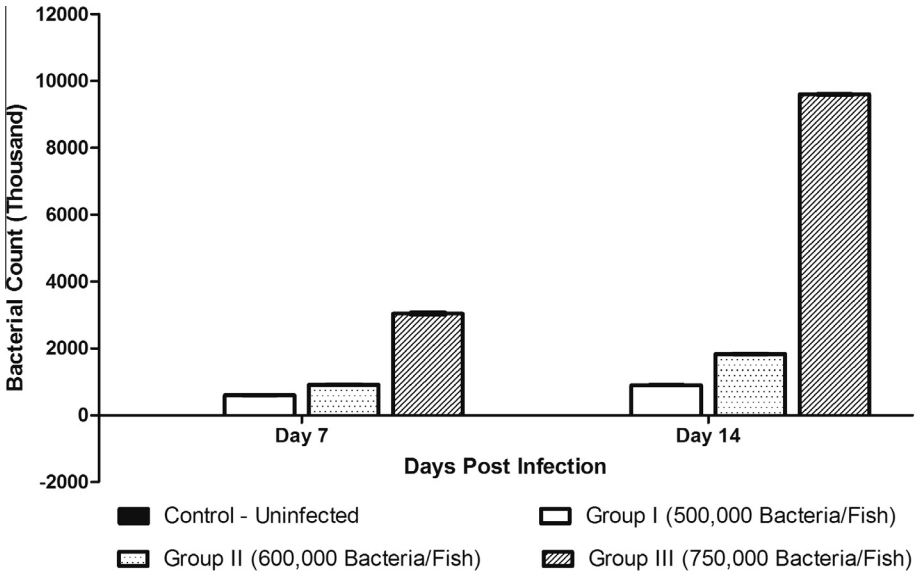

Fig. 2 – Bacterial counts of groups I, II, III (Mean ± S.E.M., $n = 6$) on days 7 and 14 during the optimization study of *M. marinum*-induced adult zebrafish model.

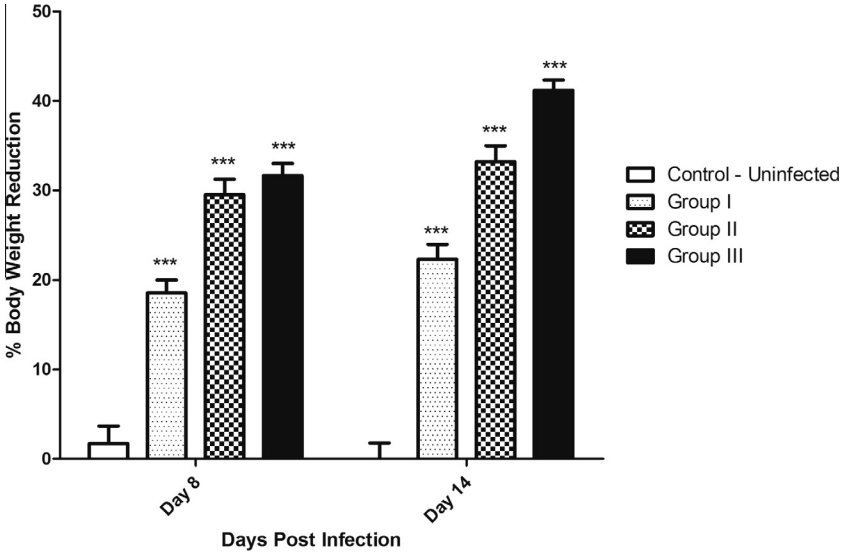

Fig. 3 – Percentage body weight reduction observed in groups control-uninfected, I, II, III (Mean ± S.E.M., $n = 10$) on days 7 and 14 during the bacterial optimization study of *M. marinum*-induced adult zebrafish model. The statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$) with respect to un-infected control group has been analyzed by one-way ANOVA using *GraphPad Prism Software*.

elimination of bacteria with a very high statistical significance.

The data on body weight reduction showed that fish treated with Moxifloxacin and the combination of Isoniazid + Rifampicin did not show any significant reduction in body weight as compared with the untreated control on day 14 of the study. Furthermore, there was a reduction in body weights for the first seven days of the infection phase which improved in the treatment phase (Fig. 6a). Fish treated with Isoniazid, Rifampicin and Ethambutol showed a statistically significant reduction in body weight as compared with the untreated control; however, the reduction seen in the infected control group and the Amoxicillin treated group was over 50%, which showed a severe infection in these fish.

Overall, the evaluation that the anti-tuberculosis could be ranked for efficacy in the following order is based on various parameters summarized in Table 1:

(a) Moxifloxacin
(b) Isoniazid + Rifampicin
(c) Rifampicin
(d) Ethambutol
(e) Isoniazid

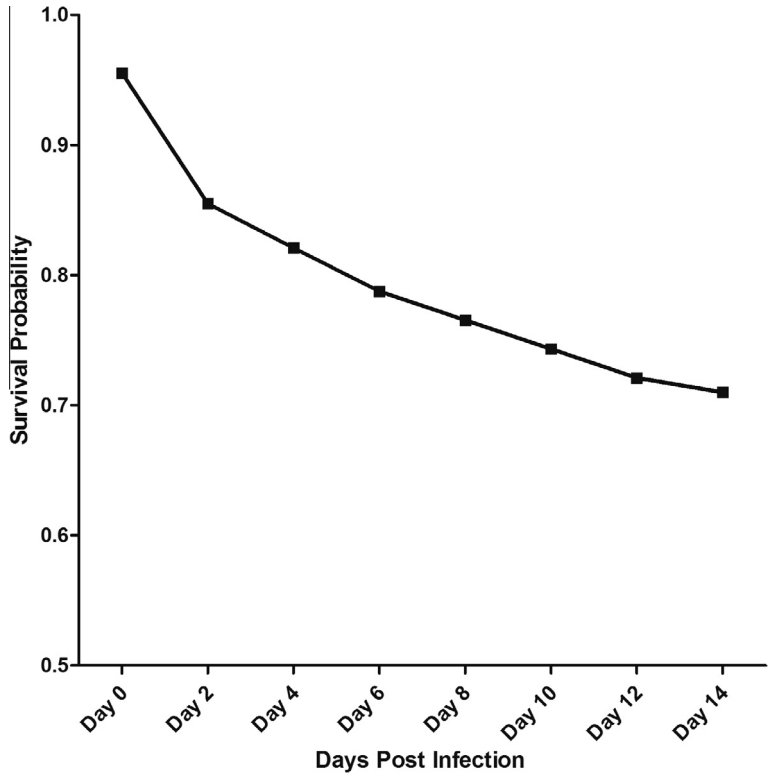

Fig. 4 – Kaplan–Meier survival analysis performed to know survival probability of Group III fish induced with 750,000 bacteria.

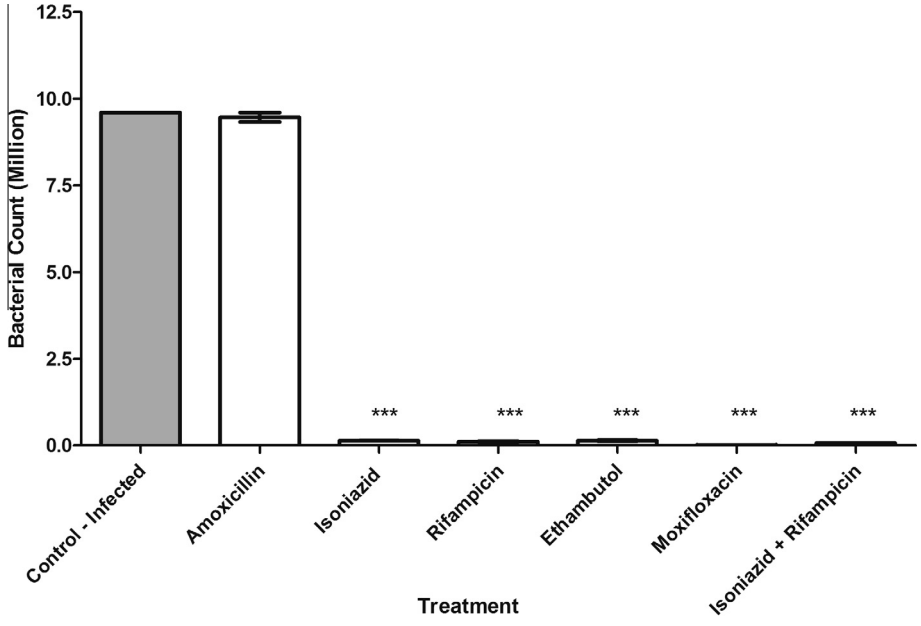

Fig. 5 – Bacterial count estimation (Mean ± S.E.M., $n = 6$) for control and treated groups conducted by using MPN (most probable number) assay. The statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$) with respect to infected control group has been analyzed by one-way ANOVA using *GraphPad Prism Software*.

It is known that Moxifloxacin and a combination therapy of Isoniazid + Rifampicin are more efficacious in human TB followed by the other three studies in this experiment. This result suggests that the efficacy profile observed in the zebrafish model is similar to the one seen in a clinical situation, demonstrating the predictive value of this model in drug screening.

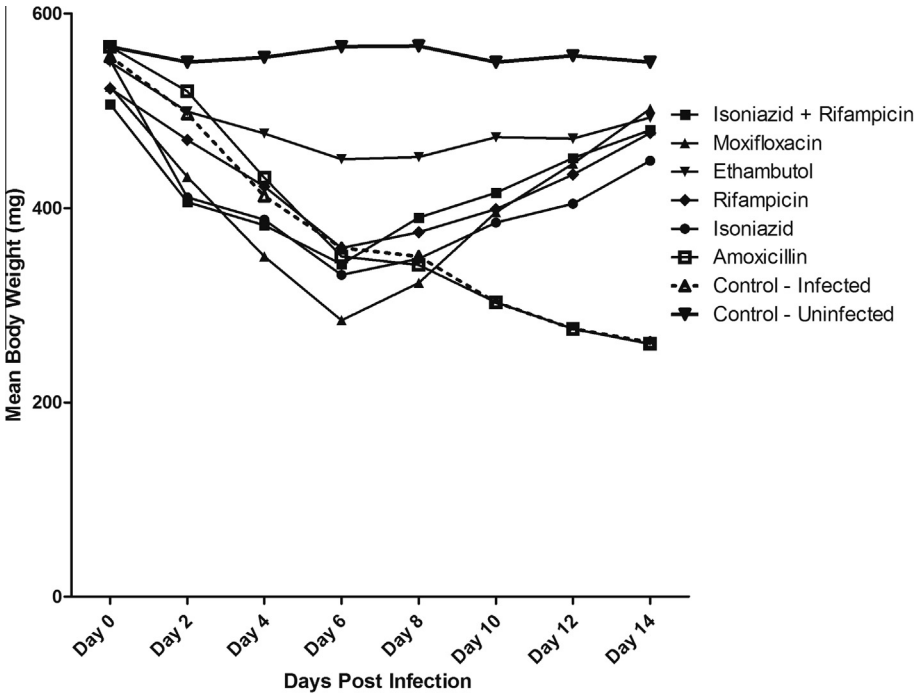

Fig. 6a – Mean body weight changes observed (Mean ± S.E.M., $n = 10$) with respective days post infection for all the groups during the study of *M. marinum*-induced adult zebrafish model.

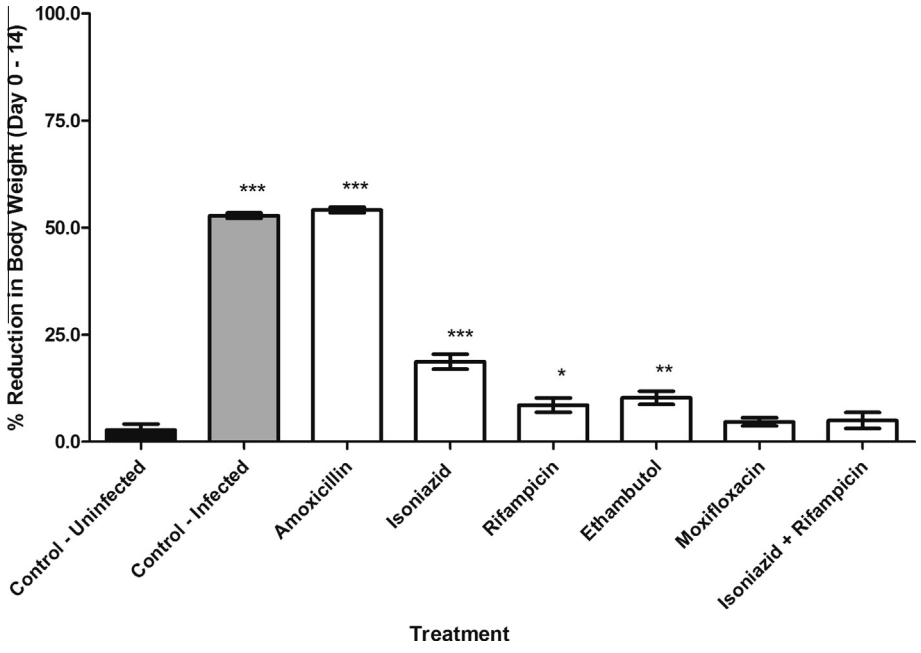

Fig. 6b – Percentage body weight reduction (Mean ± S.E.M., $n = 10$) over the study period for control and treated groups of *M. marinum*-induced adult zebrafish model. The statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$) with respect to un-infected control group has been analyzed by one-way ANOVA using *GraphPad Prism Software*.

Discussion

The use of adult zebrafish as a surrogate model for assessing drug efficacy in TB research has not been fully exploited by either academicians or by the industry. Important aspects of establishment of a screening tool for evaluation of pharmacological activity of drugs include genetics and physiology rationale, predictivity and reproducibility of protocol. The genetic and physiological relevance of *M. marinum*-induced adult zebrafish models of TB has been well established in the literature [19]. Various methods and models of *M. marinum* have been reported in the literature. A rapid high-throughput platform

Table 1 – Ranking of anti-tuberculosis drugs based on various parameters of efficacy in M. *marinum*-induced adult zebrafish model of tuberculosis.

Rank	Anti-tuberculosis drug	Dose	Parameter values (Day 14 post infection)		
			MPN (million/fish)	% Body weight reduction	Survivability
1	Moxifloxacin	5 mg/kg	0.01 ± 0.00	4.4 ± 1.0	0.6
2	Isoniazid + Rifampicin	5 mg/kg + 2.5 mg/kg	0.07 ± 0.00	5.3 ± 1.8	0.6
3	Rifampicin	5 mg/kg	0.12 ± 0.01	8.8 ± 1.6	0.7
4	Ethambutol	10 mg/kg	0.14 ± 0.01	10.3 ± 1.6	0.7
5	Isoniazid	10 mg/kg	0.14 ± 0.01	18.7 ± 1.7	0.7

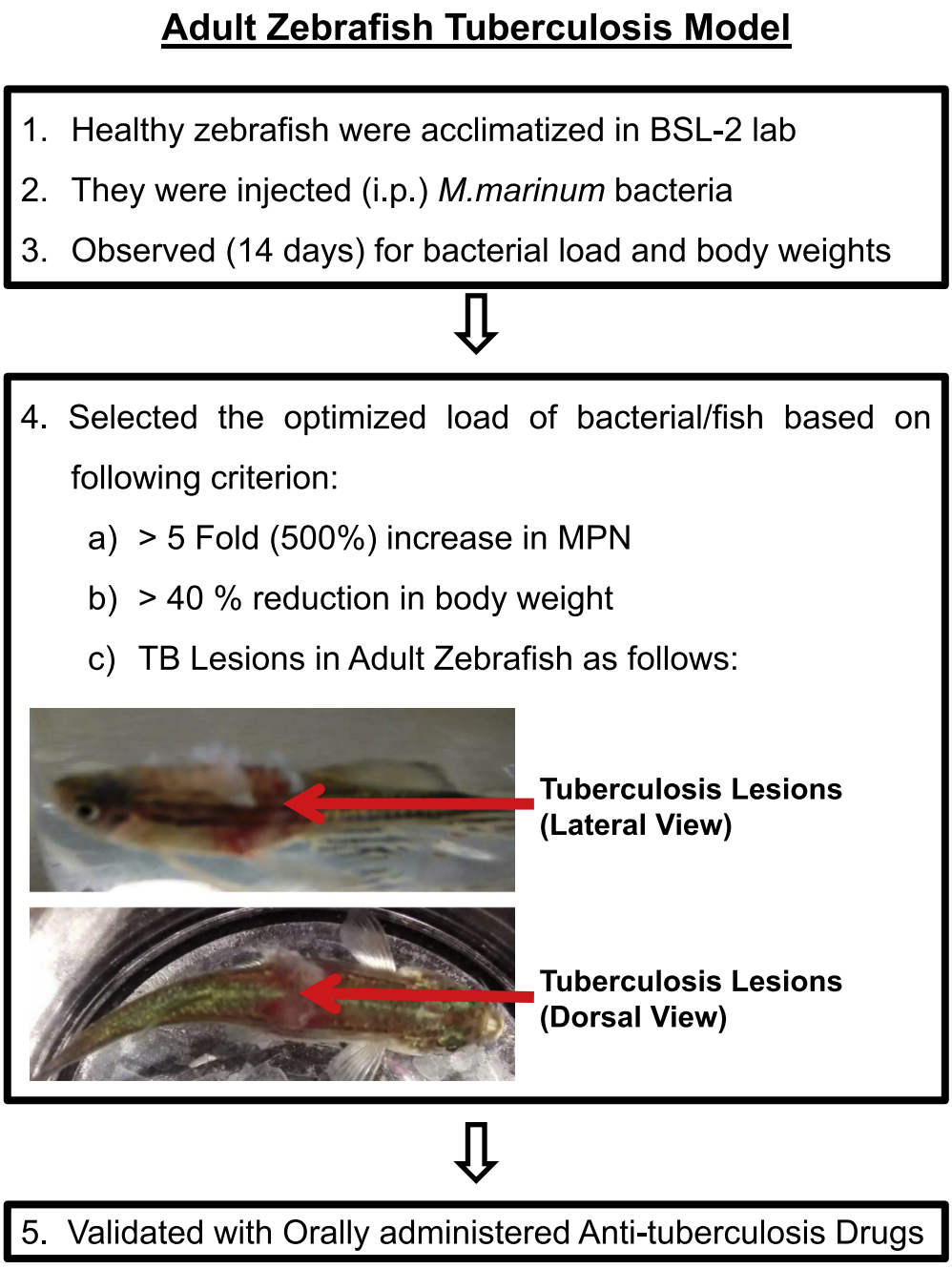

Fig. 7 – Flow chart demonstrating the standardization process followed in developing adult Zebrafish tuberculosis model for evaluation of oral anti-tuberculosis drugs.

wherein zebrafish larva infected with fluorescently labeled *M. marinum* are monitored using automated plate fluorometry (APF) has been developed to assess both efficacy and safety [20]. A similar high-throughput larval model using larval zebrafish has been reported recently [21]. The adult zebrafish model of *M. marinum* infection has been reported [2], however, the report deals with the study of pathology with respect to adaptive immunity and this model has not been validated for drug screening. The present protocol has been inspired by this report, and an attempt to modify and refine the protocol has been made in order to make it reproducible and simple to conduct.

A flow chart for standardization of the protocol for *M. marinum*-based adult zebrafish TB model has been suggested in Fig. 7. It provided for criterion for each step of standardization and validation of the model. This flow chart will be very useful for the development of such a model in laboratories across academia and the industry.

It is proposed that the use of adult zebrafish model should take precedence over the larval model (which has been more popular) based on the following rationale: adult zebrafish have optimally developed organs required for drug metabolism [22,23]; poorly soluble drugs may precipitate and will be unabsorbed in the larval assay and hence cannot be tested in larval zebrafish; using the oral dosing paradigm, the dosage of the drugs can be ascertained in terms of mg/kg. Therefore, even though the larval models can be useful for early high-throughput screening, the adult model holds greater promise for the establishment of *in vivo* proof-of-concept. An alternative to the oral method of administration, the intraperitoneal (i.p.) method can also be used for precision drug administration [24] and for arriving at a dose in terms of mg/kg. The use of the adult zebrafish model helps us have a fully grown organism with optimally functioning systems of ADME (absorption, distribution, metabolism and excretion) and precise methods for drug administration. This can ensure that the effect of candidate drugs can be determined to take decisions in preclinical research. This protocol would further help researchers to correlate drug efficacy data between zebrafish and other mammalian models.

The methods reported so far using larval zebrafish require fluorescently labeled organisms and sophisticated visualization techniques. Moreover, micro-injections for the inoculation of infection will require specific equipment and trained manpower. Small academic laboratories and start-up companies may not be in a position to make these investments for a small library-based screening program. Even larger organizations that are not interested in high-throughput screening would prefer a protocol which can be used in low-resource settings. This protocol is resource-efficient, inexpensive, involves simple techniques like bacterial count, body weight change and survivability.

Literature reports have suggested the use of zebrafish as a model organism to study various other bacterial infections like *Burkholderia cenocepacia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Francisella species*, the fungal pathogen *Candida albicans* and the viral pathogen herpes simplex virus type 1 [25–31]. It is believed that these methods can be modified suitably and applied to these infections as well.

A possible improvement in the present protocol could be the addition of pharmacokinetic assessment of test drugs in order to develop pharmacokinetic-pharmacodynamic correlation of efficacy. Drug metabolism can also be assessed in the same study. In conclusion, the use of adult zebrafish and the involvement of simple phenotypic parameters ensure obtaining the maximum data about compounds from one study, a possibility that makes this protocol very useful in drug discovery decision-making.

Contributors

Jonnalagadda Padma Sridevi and Hasitha Shilpa Anantaraju performed all the experiments, conducted data compilation and prepared the manuscript. Pushkar Kulkarni conducted data interpretation and coordinated activities related to the preparation of the manuscript. Perumal Yogeeswari and Dharmarajan Sriram contributed towards scientific discussions pertaining to all experiments and reviewing the manuscript. Pushkar Kulkarni, Yogeeswari and Dharmarajan Sriram supervised all the experiments and made necessary arrangements for resources.

Conflict of interest

We have no conflict of interest to declare

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