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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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ARTICLE INFO

Article history:
Received 1 October 2014
Accepted 2 October 2014
Available online 22 October 2014

Keywords:
Anti-tuberculosis drugs
Drug discovery
Mycobacterium marinum
Mycobacterium tuberculosis
Oral dosing
Adult zebrafish

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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http://dx.doi.org/10.1016/j.ijmyco.2014.10.001

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].