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A Contemporary Warming/Restraining Device for Efficient Tail Vein Injections in a Murine Fungal Sepsis Model

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TITLE:

A Contemporary Warming/Restraining Device for Efficient Tail Vein Injections in a Murine Fungal Sepsis Model

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Rodent tail vein injections, intravenous injections, rodent warming device, rodent restraining device, animal models, sepsis models, vasodilation

SUMMARY:
Here, we present an effective and efficient method for rodent tail vein injections using a uniquely designed warming/restraining device. By streamlining the initiation of vasodilation and restraining processes, this protocol allows accurate and timely intravenous injections of large groups of animals with minimal distress.

ABSTRACT:
In rodent models, tail vein injections are important methods for intravenous administration of experimental agents. Tail vein injections typically involve warming of the animal to promote vasodilation, which aids in both the identification of the blood vessels and positioning of the needle into the vessel lumen while securely restraining the animal. Although tail vein injections are common procedures in many protocols and are not considered highly technical if performed correctly, accurate and consistent injections are crucial to obtain reproducible results and minimize variability. Conventional methods for inducing vasodilation prior to tail vein injections generally depend on the use of a heat source such as a heat lamp, electrical/rechargeable heat pads, or pre-heated water at 37 °C. Despite being readily accessible in a standard laboratory setting, these tools evidently suffer from poor/limited thermo-regulatory capacity. Similarly, although various forms of restraining devices are commercially available, they must be used carefully to avoid trauma to the animals. These limitations of the current methods create

unnecessary variables in experiments or result in varying outcomes between experiments and/or laboratories.

In this article, we demonstrate an improved protocol using an innovative device that combines an independent, thermally regulated, warming device with an adjustable restraining unit into one system for efficient streamlined tail vein injection. The example we use is an intravenous model of fungal bloodstream infection that results in sepsis. The warming apparatus consists of a heat-reflective acrylic box installed with an adjustable automatic thermostat to maintain the internal temperature at a pre-set threshold. Likewise, the width and height of the cone restraining apparatus can be adjusted to safely accommodate various rodent sizes. With the advanced and versatile features of the device, the technique shown here could become a useful tool across a range of research areas involving rodent models that employ tail vein injections.

INTRODUCTION:

The use of animal models involving rodents has been a staple of biomedical research. Numerous inbred and outbred strains, as well as genetically modified lines, are available and routinely used in laboratories worldwide. Tail vein injection is one of the essential methods in rodent models requiring intravenous (i.v.) administration of experimental agents. Generally, i.v. injections have major advantages over other routes of administration such as high absorbance rates by bypassing local tissues and the digestive tract and high tolerance to solutions of a wide range of concentrations or non-physiological pH[1-4]. Among other viable i.v. routes (e.g., saphenous veins, retro-orbital venous sinus), tail veins are considered the safest and most readily accessible blood vessel in rodents[2,3,5,6]. Hence, tail vein injection has been widely employed in an array of rodent models including infectious disease models[7-9], transplantation of biological materials[10,11], evaluation of preclinical therapeutics[12,13], and toxicological analyses[14,15].

Consistency and accuracy of dosing are a critical requirement in successful tail vein injections. Surprisingly, quantitative and qualitative evaluation of tail vein injections in the literature implicates frequent mis-injections[16,17]. A study reported that twelve out of thirty injections performed by trained injectors left more than 10% of injected doses within the tail[18]. In addition, the safety and comfort of the animal receiving tail vein injections should be a primary concern during the procedure. Improper restraint can lead to injuries and a range of stress-related pathologies (e.g., weight loss, impaired immune responses) that could introduce substantial variables in sample quality[19,20]. These errors can cause increased variability in data and poor reproducibility, thus negatively affecting study outcomes.

Induction of vascular dilation in the animal is often necessary when performing tail vein injections due to the small diameter of the vessel, estimated to be 300 µm in mice[21]. Vasodilation enhances the visibility of tail veins and aids in achieving optimal needle-vein alignment within the venous lumen. A variety of methods have been reported by laboratories such as immersing the tail in warm water[22], applying heat to the tail using a warm drape, lamp, or hair dryer[23,24], or placing the animal in a warm environment using a heating pad, incubator, or box combined with one of these heat sources[25]. The devices can be either self-made for specific purposes or available from commercial suppliers. However, many lack thermoregulatory capabilities and if any, the device

temperature is poorly maintained and often subject to variations in room temperature. Similarly, the use of a restraining device is necessary for tail vein injections as the use of anesthesia is not recommended[26,27]. Several types of laboratory-specific or commercial restraining devices have been developed. Typically, the animal is placed in a disposable 50 ml conical tube[4], slotted plexiglass walls, a tunnel, or cone[28], all of which allow ample exposure of the tail while restricting movements of the animal. However, most restrainers have size limitations due to the rigidity of the materials. Furthermore, modern high-complexity devices, despite the practical and sophisticated designs, do not appear to be feasible for injections involving large groups of animals[22].

Mouse models of bloodstream infection and associated sepsis are a prime example of situations requiring the use of this technique. Among all microbial etiology of severe clinical sepsis, fungal sepsis is often a fatal condition with mortality rates of >40% despite antifungal therapy[29]. In fact, infection by *Candida albicans* has been reported as the fourth leading cause of hospital-acquired bloodstream infection (candidemia)[30,31]. In intra-abdominal candidiasis, microorganisms in the gastrointestinal tract can disseminate via the bloodstream and cause polymicrobial sepsis with an even greater mortality[32-34]. As most nosocomial candidemia cases emerge from contaminated central line catheters or indwelling medical devices[35,36], i.v. inoculation with *C. albicans* by tail vein injection can closely mirror human sepsis development and has been a staple method in a mouse model of hematogenously disseminated candidiasis[37,38]. In this model, mortality that occurs in days can be extended or shortened by adjusting the *C. albicans* i.v. inoculum[39-41].

Recently, our laboratory has developed an innovative protocol for an optimally streamlined tail vein injection using an innovative device equipped with a thermoregulated warming unit, paired with an adjustable restraining unit, in one convenient system. This protocol allows researchers to perform tail vein injections in an accurate and timely manner, while animals can be safely conditioned and restrained for the procedure with minimal distress. The techniques demonstrated here, with the use of the advanced warming and restraining device, could serve as a useful tool in various research areas employing rodent models.

PROTOCOL:

All animal protocols involving tail vein injections and use of the warming/restraining device were reviewed and approved by the local Institutional Animal Care Committee (IACUC).

1. **Preparation**

1.1 Acclimate animals in the housing environment for at least 1 week, and allow food and water ad libitum.

NOTE: For most new users of this injection technique, animal strains with white or light-colored fur may be preferable as the tail veins are readily visible through the skin. Dark-colored strains of mice (e.g., C57BL/6) or rats (e.g., Brown Norway) have deeply pigmented tails, resulting in a weak color contrast against the vein. It is highly recommended that new users receive adequate

training until proficiency is attained.

1.2 Agents for tail vein injection

1.2.1 Prepare all test agents and solutions aseptically. When administering organisms or cellular materials, take precautions during all steps of processing to maintain pyrogen-free conditions.

1.2.2 Use only normal saline (0.9% w/v sodium chloride) or balanced salt solutions such as phosphate-buffered saline (PBS) as vehicles for tail vein injection.

CAUTION: Never use water, oil, or viscous solutions owing to the potential risk of vascular damage. A wide range of pH (4.5–8.0) is tolerable because of the buffering effect of blood and fast blood flow rates in rodents. However, highly acidic or alkaline solutions can result in unnecessary tissue damage at the injection site and should be avoided.

1.2.3 Limit the volume and frequency of injection to a minimum. Use the recommended volumes for mice and rats (≤200 µL and ≤500 µL, respectively) at body temperature before injection to minimize stress to the animal[3].

1.2.4 Ensure that each preparation of the syringe and needle is free from air bubbles in the solution; if bubbles are present, purge them completely to prevent the risk for embolism.

NOTE: Typically, 1 mL syringes with 27 G, ½-in needles are adequate for most tail vein injections.

1.2.5 Use appropriate personal protective equipment (PPE) required by local IACUC with the minimum of disposable or dedicated gowns and latex or nitrile gloves. Use safety glasses when performing tail vein injection.

1.3 The warming and restraint device

1.3.1 Carefully inspect all components before use to ensure that the device is free of any defects (**Figure 1**).

1.3.2 Warming device initialization (**Figure 2A**)

1.3.2.1. Place the warming unit on a clean flat benchtop, and power the device on. Ensure that the thermostat power indicator lamp is lit green. Place bedding materials inside the warming chamber to keep the area dry and retain heat.

1.3.3 Restraint device setup (**Figure 2B**)

1.3.3.1. Place the restraint unit alongside the warming unit, and determine the appropriate cone sizes for the animal. If necessary, manually adjust the base widths of the pliable aluminum cone

to provide adequate restraint for the animal. Alternatively, replace the cone with custom-fitted models to accommodate mice or rats of varying body sizes.

2. Tail vein injection

2.1 Apparatus adjustments

2.1.1 Setting the internal temperature

2.1.1.1. Using the control dial, set the thermostat at the desired temperature. Ensure that the heater indicator is lit red, and that the light bulb illuminates. Monitor the internal temperature display carefully while the bulb is illuminated (heating). The thermostat inactivates the bulb automatically once a target temperature has reached, approximately in 10–15 min.

NOTE: Setting a temperature higher than the ambient temperature activates the heater. In general, the recommended housing temperature in standard vivarium conditions vary, ranging from 20 to 26 °C, while the neutral (i.e., comfortable) temperature for laboratory mice is considered to be between 30 and 32 °C[42]. Therefore, it is recommended that the internal temperature of the warming chamber be elevated slightly higher than thermoneutrality, approximately at 32–36 °C. Never set the thermostat above the body temperature.

2.1.2 Positioning the restraint platform

2.1.2.1. Using the height adjustment knob, adjust the cone height to the optimal level for the user.

2.2 Heat treatment (**Figure 3A**)

2.2.1 Once the target temperature has been reached (32–36 °C), gently transfer the animals from the housing cage into the warming chamber.

NOTE: Heat treatment for 5–10 min is sufficient to induce vasodilation and enhance the visibility of tail veins. However, animals can be safely held in the thermoregulated chamber for the duration of the procedure (typically 20–30 min with no sign of hyperthermia). The warming chamber can safely contain 4–6 mice or one rat.

2.2.2 Monitor the animal for any signs of acute heat stress (e.g., rapid respiration, lethargy, jumping escape behavior).

CAUTION: Animals exhibiting signs of hyperthermia should be returned to their cage and monitored until they resume normal activity prior to reuse. If this is because of the internal temperature exceeding the optimal range, ensure that the warming device is turned off.

2.3 Injection steps

2.3.1 Lift the animal by the base of the tail, and remove it from the warming chamber. Introduce the animal onto the cone opening of the restraining unit.

CAUTION: Never lift mice from the tail end; this can result in serious injuries. Alternative methods of handling should be used for obese or pregnant mice[28].

2.3.2 As the animal grasps on the far edge of the cone with its forelegs, gently pull the tail backward and pass the tail through the open slit. Secure the hind end of the animal at the base of the cone with one hind leg protruding out from the cone so the lateral vein is shown at a position of 12 o'clock. Either hind leg can be protruded as there are two lateral veins, one on each side (**Figure 3B**).

2.3.3 Grasp the tail at the mid- to two-thirds-length with the non-dominant hand between the thumb and forefinger, putting slight tension on the lateral vein to maintain the tail positioning and vasodilation.

NOTE: Enhanced visibility of the dilated veins by heat treatment enables the user to quickly determine an injection site for the best results (**Figure 4**).

2.3.4 Wipe the skin of the injection site with a gauze sponge or pad moistened with 70% alcohol. Clean as gently and quickly as possible to avoid irritation to the tail.

NOTE: This procedure can be omitted at the discretion of the institutional IACUC.

2.3.5 Hold the syringe with the dominant hand, and position the needle parallel to the tail. Insert the needle toward the direction of the blood flow, bevel up at a 10–15° angle (**Figure 5A–B**), and advance further into the lumen of the vein by penetrating 2–4 mm (**Figure 5C–D**). Slowly inject the solution.

NOTE: If the injection is successful, no resistance on the plunger should be felt, and the fluid can be seen moving through the vein. In case of resistance or white blisters above the injection site, remove the needle and attempt a second injection at a site above the original needle placement. Do not attempt to inject below the initial injection site as the fluid will release through the initial site. If injection of one lateral vein is unsuccessful, reposition the animal to the opposite side and make more attempts on the contralateral vein. The maximum number of attempts will depend on where one starts the attempted injection along the vein and the swelling that may occur with missed attempts. Consult the institutional IACUC regulations for mis-injections and associated injuries.

2.3.6 Remove the needle, and press firmly with the thumb to prevent backflow of the injected solution and/or blood. Continue to apply gentle compression with a clean gauze/wipe or tissue until bleeding has stopped (**Figure 6**).

2.3.7 Return the animal to its cage, and monitor for at least 5 min. Ensure that the animal resumes normal activity without further bleeding.

3. A murine model of fungal bloodstream infection and sepsis

3.1 Mouse strains

3.1.1. Acclimate female Swiss Webster outbred mice at 6 weeks of age per institutionally recommended guidelines. Alternatively, use inbred/genetically modified strains (e.g., C57BL/6 background) for this protocol with modified inocula (see NOTE).

NOTE (see Discussion for detail): Tail veins of mice with dark fur are often less visible than those with lighter fur due to the deeply pigmented tail (**Figure 4**). There is varying susceptibility to fungal sepsis/lethality among different mouse strains. Use of mouse strains other than Swiss Webster may require additional protocol optimization by considering relevant factors (e.g., genetic background, age, sex, body size) that could influence host immune status. For example, a lethal challenge in C57BL/6 mice typically requires higher inocula (up to 10x) to achieve the level of mortality seen in Swiss Webster mice.

3.2 Microorganisms

3.2.1. For a lethal challenge (sepsis), streak frozen stocks of *Candida albicans* strain DAY185 (or strains of choice) onto Sabouraud dextrose agar and incubate at 30 °C for 2 days.

3.2.2. Transfer a single colony into 10 mL yeast extract-peptone-dextrose broth, and culture to the stationary phase of growth for 18 h at 30 °C with shaking.

3.3 Inoculum solutions

3.3.1. On the day of a lethal challenge, collect the broth culture, and wash the pellet 3 times by centrifugation (800 × *g*) in sterile PBS.

3.3.2. Identify viable yeast cells by trypan blue dye exclusion, and enumerate using a hemocytometer. Adjust the cell concentration to 1 x 10^6 cells/mL in sterile PBS at room temperature.

NOTE: Each animal will receive 100 µL of the inoculum solution. Prepare an excess volume of the inoculum (>500 µL) to allow for potential loss during the injection procedure. The final inoculum is 1 x 10^5 cells per mouse. The inoculum volume can be increased up to 200 µL by adjusting the cell concentration accordingly.

CAUTION: The fungal inoculum solution must be kept at room temperature prior to injection. Warming the inoculum solution to body temperature may induce a morphological change from yeast cells to hyphae. Contrarily, bolus i.v administration of cold solutions can rapidly lower body

temperature of the animal and should be avoided.

3.4 Intravenous inoculation

3.4.1. Warm the animals, and induce vasodilation by following the procedures in section 2.

3.4.2. Inject 100 µL of the inoculum solution into the tail vein using a 1 mL syringe with a 27 G, ½-in needle.

3.5 Post-inoculation monitoring

3.5.1. Monitor the animals for the following signs of sepsis-induced morbidity: i) fur aspect (e.g., smooth, ruffled), ii) activity (e.g., moving freely, nonresponsive), iii) posture (e.g., hunched, stiff), iv) behavior (e.g., slow, no relocation), v) chest movements (e.g., normal breathing, dyspnea), vi) eyelids (e.g., open, closed)[43].

3.6 Sepsis scoring

3.6.1. Score the observed morbidity according to a modified Mouse Clinical Assessment Score for Sepsis (M-CASS) in a four-point grading scale from 0 to 3 in each category: 0, normal; 1, mild; 2, moderate; 3, severe[43].

3.7 Optional protocol: Vaccination against fungal sepsis

3.7.1 Fourteen days prior to a lethal challenge, inoculate mice with *Candida dubliniensis* strain Wü284 or attenuated *C. albicans* strains, such as Δ*efg1*/Δ*cph1* mutant ($1x10^5$ cells per mouse), as described in sections 3.2–3.4 in lieu of *C. albicans* DAY185.

3.7.2 Conduct a lethal challenge in the vaccinated mice, as described in sections 3.2–3.4, and monitor for the signs of sepsis-induced morbidity described in sections 3.5–3.6.

REPRESENTATIVE RESULTS:

The temperature inside the warming chamber is continuously detected by the internal sensor and auto-regulated by the thermostat. First, the control dial of the thermostat was positioned at 78, 85, 90, or 95 °F (26, 29, 32, or 95 °C) to select set temperatures. Once the heater was activated (**Figure 7**, yellow dots), heat emission by the light bulb rapidly raised the internal temperature during the first 5–15 min, depending on the set temperature. The heater inactivated the light bulb if the detected internal temperature exceeded the set temperature (gray dots). The initial peak temperatures should rise to 5–7 °C above the set temperatures in all groups to offset the temperature loss during animal transfer. Subsequently, the device continues to repeat the heat cycle automatically and maintains the warming chamber at the set temperature.

An example of experimental data obtained by successful tail vein injections using the current protocol is shown in **Figure 8**. In a mouse model of bloodstream candidiasis resulting in sepsis,

an i.v. challenge with *Candida albicans* (1 x 10^5 cells per mouse) in Swiss Webster mice caused a rapid onset of sepsis and dissemination of the organisms, leading to high mortality within 3–4 days (open dots) (**Figure 8A**). In contrast, animals could be protected from sepsis by prior i.v. pre-immunization/vaccination with an avirulent yeast strain, *Candida dubliniensis*, achieving >95% survival following the lethal i.v. challenge with virulent *C. albicans* (solid dots). These results in progressive mortality vs. vaccine-mediated protection were obtained reproducibly in four independent experiments (**Supplemental Figure 1**). Similar protection could be achieved using other avirulent yeast strains such as attenuated *C. albicans* mutants (Δ*efg1*/Δ*cph1)* (data not shown). Sepsis could be monitored as well and correlated to mortality; the unvaccinated animals with lethal infection had a significant increase in sepsis-induced morbidity, whereas the vaccinated group exhibited minimal symptoms following the lethal challenge (**Figure 8B**).

FIGURE AND TABLE LEGENDS:

Figure 1. Description of the rodent warming and restraining device. (A) shows the exterior view of the warming device, which consists of:

1 Thermostat cover – lift upward by the handle to expose the thermostat
2 Electrical enclosure – sealed permanently for protection
3 Chamber lid – lift upward during animal transfer to/from
4 Warming chamber – removable, cover the floor with bedding before use
5 Restraint apparatus – stowable with the heating device while not in use
6 Power switch – inline rocker switch for main on/off functions
7 Power cord – voltage/current: 120V/10A

(B) shows the interior of the warming device:

8 Incandescent light bulb – light output at 100 Watts
9 Light bulb protective shield – removable for bulb replacement
10 Temperature sensor probe – located inside the chamber
11 Internal temperature thermometer – place inside the chamber for monitoring temperature

(C) shows components of the warming device thermostat:

11 Internal temperature thermometer
12 Thermostat – auto-regulates the heater
13 Setpoint control lever – minimum/maximum: 78 °F/108 °F (25 °C/42 °C)
14 Thermostat power indicator – green light indicates normal operation
15 Thermostat heater indicator – lit red during heating cycle

(D) shows components of the restraining device:

16 Cone – pliable aluminum sheet designed for rodent restraint
17 Tail channel – shaped to permit smooth positioning of the tail
18 Cone lift platform – provides sturdy lift of the cone base
19 Height adjustment knob – designed for manual height adjustment
20 Scissor jack – height range from 45–140 mm (1.77-5.52”)

21 Support plate – installed with rubber feet to provide stability

Figure 2. The rodent warming and restraining device. (**A**) Prior to use, the two parts of the device are placed side by side on a clean bench top. (**B**) Once the warming device is powered on, the thermostat activates the heater. The light bulb remains lit and emits heat until the warming chamber has reached the set temperature. The warming device automatically repeats the heat cycle to maintain the internal temperature.

Figure 3. Mice (C57BL/6) placed in the warming and restraining device. (**A**) Mice receiving heat treatment for vasodilation. The animals (4–6 mice per treatment) are transferred from their housing cage into the warming chamber of the device and heat-treated for a minimum of 5–10 min. (**B**) A mouse restrained for tail vein injection. The mouse is transferred from the warming chamber into the cone opening of the restraining device with its tail passing through the open slit. The mouse is gently pulled backward to the far edge of the cone until the base of the tail reaches the tip of the cone. As the animal is drawn toward the base of the cone with a gentle lateral rotation, one hind leg is positioned upward so that it protrudes out from the open slit allowing the lateral tail vein to be positioned at 12 o'clock.

Figure 4. Identification of the lateral tail veins in mice. (**A**) The tail of an untreated Swiss Webster mouse. The mouse is placed in the restraining device without prior heat treatment for vasodilation. The lateral tail vein can be identified as a thin dark vessel that courses under the skin. (**B**) The tail of a Swiss Webster mouse treated with the warming device for 10 min. The heat-treated mouse is restrained for tail vein injection. The lateral tail vein is readily visible through the skin due to the enlarged vessel diameter induced by vasodilation. (**C**) The tail of a C57BL/6 mouse treated with the warming device for 10 min and restrained for tail vein injection. Vasodilation enhances visibility of the tail vein through the deeply pigmented skin although the vein is not as readily visible as in the light-colored Swiss Webster mice due to a weak color contrast against the vein. Red arrows denote the location of the lateral tail vein.

Figure 5. Tail vein injection performed in heat-treated mice (Swiss Webster). (**A–B**) Needle insertion into the lateral tail vein at the injection site. The needle (27 G, ½-in) is positioned parallel to the tail vein with the bevel up and pointed toward the blood flow and inserted. (**C–D**) Needle placement in the tail vein and injection. The tip of the needle is further advanced 2–4 mm into the lumen of the vein. The thumb is positioned on the plunger of the syringe, and the desired volume is dispensed with slow and steady pressure. Oval circles indicate injection sites.

Figure 6. Post-injection procedure. (**A**) A bleeding area at the injection site. Bleeding and backflow of the injected solution occur immediately after needle removal. This can be minimized by applying firm compression on the injection site with the thumb. (**B**) Blood clot formation at the injection site. Gentle compression with a clean gauze/wipe facilitates blood clotting on the injection wound. Arrows denote injection sites.

Figure 7. The internal temperature of the warming chamber during use. The warming device was activated for warming at the designated set temperatures. The warming chamber of the

device was monitored for the internal air temperature and heat cycles (light bulb on/yellow dots, off/gray dots) were recorded over 45 min. The orange area indicates the optimal temperature range for induction of vasodilation in rodents.

Figure 8. Sepsis mortality vs. vaccine-mediated protection following a lethal challenge with *Candida albicans*. Mice (8 week-old Swiss Webster females) were vaccinated intravenously with avirulent live *Candida dubliniensis* Wü284 (*Cd*), followed by a lethal intravenous challenge with wild-type *C. albicans* DAY 185 (1 x 10^5 cells per mouse) 14 days later. (**A**) Mortality was assessed over 10 days following the lethal challenge. (**B**) Animals were monitored for sepsis morbidity and scored according to a modified Mouse Clinical Assessment Score for Sepsis (M-CASS)[43]. Data are cumulative of 4 independent experiments with 10 mice per group and analyzed using the Mantel-Cox log-rank test. **** $p < 0.0001$. SEM, standard error of the mean.

Supplemental Figure 1. Reproducibility of sepsis mortality and vaccine-mediated survival from a bloodstream *Candida albicans* challenge. Each panel represents data from four independent experiments included in a cumulative result shown in **Figure 8A**. Each experiment was conducted using 10 mice per group and analyzed using the Mantel-Cox log-rank test. *Ca, Candida albicans. Cd, Candida dubliniensis.* **** $p < 0.0001$. *** $p < 0.01$.

DISCUSSION:

Consistent and accurate dosing are key requirements for experimental reliability in animal models. This is especially important in cases of i.v. administration where systemic bioavailability of injected agents is considerably higher/faster than with other administration routes[3]. Thus, errors in tail vein injection could have a detrimental impact on study outcomes. Historically, intraperitoneal (i.p.) injection, rather than i.v., has been the most common method for systemic access in rodents due to technical simplicity and convenience. However, administration routes become more crucial when translating preclinical readouts from animals into clinical settings. Hence, there is a need for continuous improvement in rodent protocols that could facilitate successful tail vein injection.

The key advancement in the present protocol is the innovative thermoregulated warming device that enables effective induction of vasodilation in rodents, which dramatically improves the visibility of tail veins and needle alignment. Heating methods that are poorly thermoregulated (e.g., lamps), topical vasodilators or skin irritants (e.g., xylenes) are not only unreliable, but are also unsafe for the animal and should be avoided[44]. Contrary to other conventional methods, such as immersing the tail in warm water, the autoregulation capability of this device can safely condition multiple animals simultaneously. In addition, this protocol is strengthened further by using the optimally designed restraining device and allowing fast and secure immobilization of the animal in a position that best displays the lateral tail vein.

The transparent tubal formats seen in many current restrainers, though practically well-designed, require more handling time with each animal, thus prolonging the restraining process[45]. This can be more problematic in rodent strains with aggressive traits that offer limited cooperation[46,47]. In contrast, the semi-enclosed cone structure of the restraining device permits quick positioning

of the animal and aids in minimizing the duration of restraint. Together, the streamlined protocol using the innovative, highly optimized warming/restraining system accelerates the injection procedure, allowing for quick and effective dosing of large groups of animals. In our laboratory, we typically complete an entire injection procedure of 30 mice from heat treatment to post-injection monitoring within 1 h using this protocol.

Despite the advanced features, this device has some apparent disadvantages: the first is the cost of the device and routine light bulb replacement in the warming chamber. However, in addition to the efficiency and speed of injections, the device is durable for repeated use and compatible with most common disinfectants, permitting thorough cleaning of the device between uses. Together, this offsets the initial investment. Second, in situations with limited workspace, a drawback to this protocol may be the requirement for a dedicated bench area large enough to place the two units, side by side, while performing the injection. However, because the device can be utilized broadly across several rodent protocols involving i.v. injections, it is possible that the device could serve as a core instrument similar to other communal vivarium equipment such as isoflurane vaporizers. Regardless, the two units are easily portable and can be bundled and stowed while not in use.

The i.v. lethal challenge model of murine fungal sepsis described in this protocol closely mimics *C. albicans* bloodstream infections in humans and has been extensively used to study fungal virulence, test efficacy of antifungal therapies, and characterize host immune responses to infection[37,39,48]. To achieve a reproducible infection, i.v. inoculation via tail vein injection is the most vital step of the protocol to ensure accurate delivery of the organisms into the bloodstream. In fact, animals respond very differently to varying levels of *Candida* i.v. challenges; administration of too low amounts of inoculum will result in unwanted spontaneous recoveries, whereas animals receiving too high doses will succumb prematurely. The specific window of inoculum sizes for a given organism to induce a consistent level of sepsis/mortality largely depends on both fungal strains and mouse strains.

The current protocol using Swiss Webster mice at the inoculum of 1×10^5 wild-type *C. albicans* reproducibly induced the onset of sepsis morbidity within 1 day, followed by progressive mortality resulting in 100% lethality by 5–7 days. In contrast, inocula higher than 1×10^5 led to accelerated deaths (i.e., 1–2 days at 1×10^6, 3–4 days at 5×10^5), and those lower than 1×10^5 were sub-lethal. In line with numerous reports in the literature, the use of non-*albicans Candida* species in lieu of *C. albicans* resulted in significantly diminished lethality[40,49]. Additionally, the choice of mouse strains, or even the origin of colonies, can have a considerable impact on infection outcomes due to varying susceptibilities between mouse strains, as reported by others[39-41,50-55]. Hence, both should be taken into consideration when designing experiments.

Following a lethal i.v. challenge, fungal cells spread rapidly through the bloodstream and begin to invade multiple organs, among which the most affected are the kidneys[41]. Other organs affected are the brain, spleen, and bone marrow[48,56]. Regardless, acute sepsis is the ultimate cause of death at the early time points[37]. As shown in the representative results, sepsis severity can be quantitatively assessed by the Mouse Clinical Assessment Score for Sepsis (M-CASS) based

on exhibited signs of a sepsis condition in challenged animals[43,57]. Among the several surrogate markers of lethal sepsis, hypothermia has been suggested as a critical predictor for imminent death in both clinical and experimental sepsis[43,58,59].

Although no formal studies have been conducted to directly compare inbred vs. outbred mice in this model, data obtained from the current protocol using outbred Swiss Webster mice are exceptionally reproducible in various sepsis parameters, despite the presumed genetic heterogeneity. Generally, a pattern of mortality that falls within 3–5 days is a firm model of acute sepsis, as evidenced by rapid elevation in sepsis morbidity and levels of inflammatory markers within hours of post-lethal challenge[50,51]. For longer survival times (7–10 days), mortality is likely the result of microbial burden leading to lethal tissue damage in target organs and the central nervous system. The choice of sepsis or microbial burden can be applied as necessary for evaluating immune functions or responses to anti-inflammatory regimens or antifungal therapies/vaccines, as determined by the inoculum used.

In addition to the i.v. lethal challenge model, intra-abdominal infection with *C. albicans* in mice via an i.p. challenge can also lead to disseminated candidiasis and subsequent sepsis, although co-inoculation with the bacterial pathogen, *Staphylococcus aureus,* synergistically enhances mortality compared to *C. albicans* mono-infection[51,60,61]. In the i.p. lethal challenge model, substantially higher microbial inocula (1.75×10^7 *C. albicans*/8×10^7 *S. aureus* per mouse) were required to cause polymicrobial peritonitis and dissemination of the organisms from the abdominal cavity into the bloodstream. Similarly, gastrointestinal infection with *C. albicans* in mice treated with immunosuppressive and/or mucosal-damaging agents led to translocation of the fungal cells into the bloodstream and results in fungal sepsis[62,63]. Despite the distinctive inoculation routes, the mechanism of ensuing fungal sepsis was largely analogous between the three disease models, involving an uncontrolled systemic proinflammatory response to *Candida* that led to organ failure[37,51,61]. Similarly, in humans, it is this process of the host response, not simply candidemia, that causes the high morbidity/mortality associated with hematogenously disseminated candidiasis acquired in health care settings[64,65].

Using the current fungal sepsis model, we demonstrate here that protection against lethal *C. albicans* infection can be achieved by i.v. pre-immunization/vaccination with *C. dubliniensis* (avirulent) or attenuated *C. albicans* mutants, concomitant with significant reduction in sepsis morbidity. The protection is mediated by innate Gr-1^+ myeloid-derived suppressor cells that appear to be induced in the bone marrow as a form of trained innate immunity[66,67]. Efforts are underway to extend the understanding of this novel form of innate immune-mediated protection against *C. albicans* bloodstream infections.

In conclusion, the innovative rodent warming/restraining device has been instrumental in advancing our ability to perform i.v. injections of large-scale multi-group animal studies in an efficient and effective manner. As such, we have coined the term, Mouse a Minute, for the device. The device specifications are available from the corresponding author upon request for procurement of a similar device. The techniques demonstrated here could serve as a useful tool in rodent models employing tail vein injections across a broad range of research areas.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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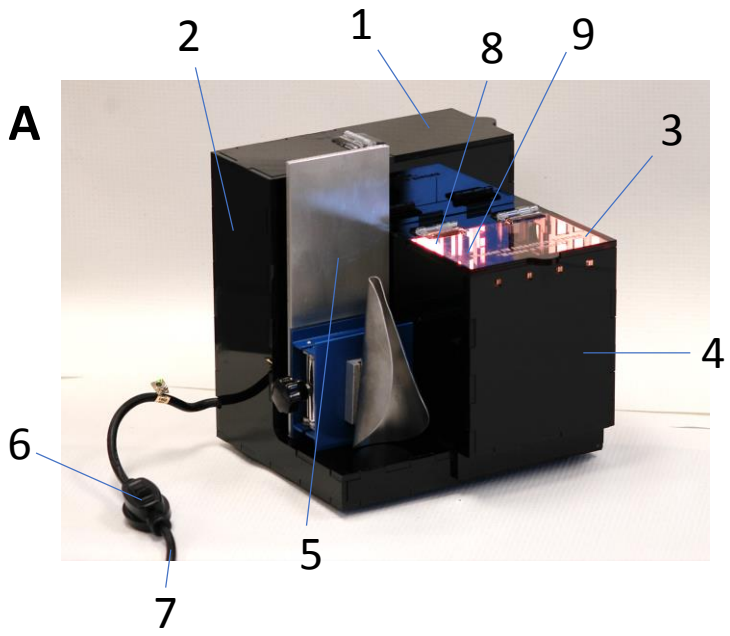
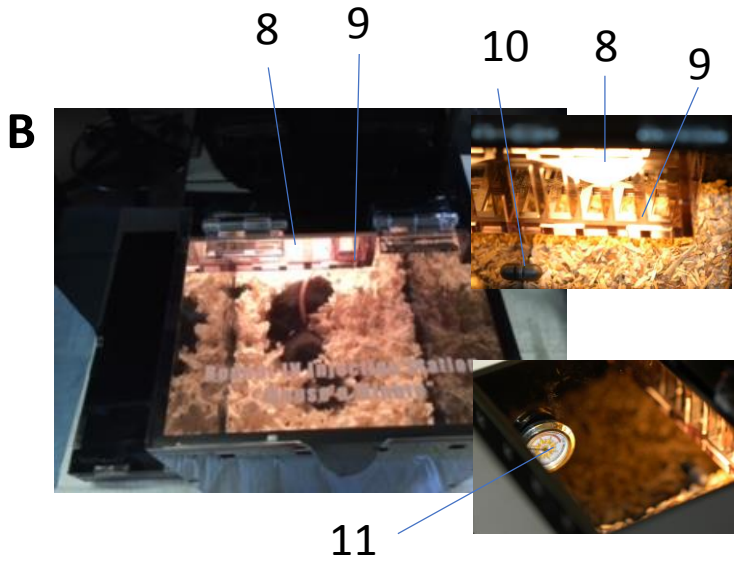
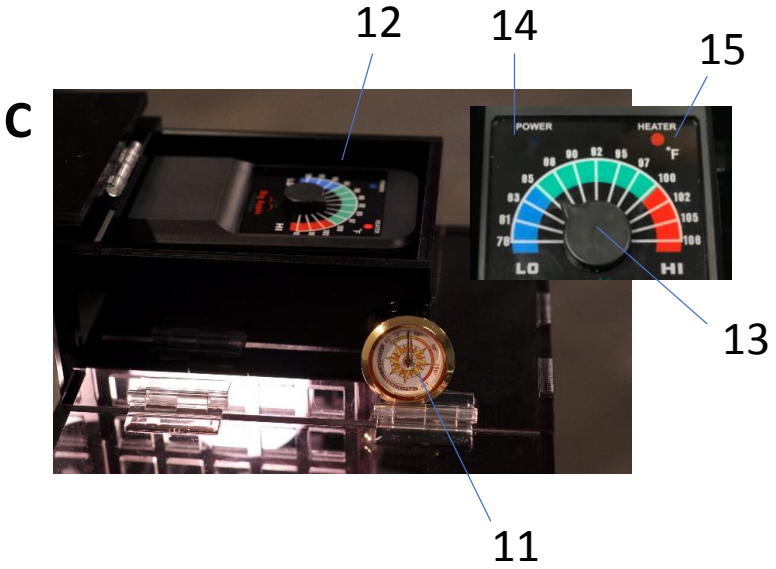
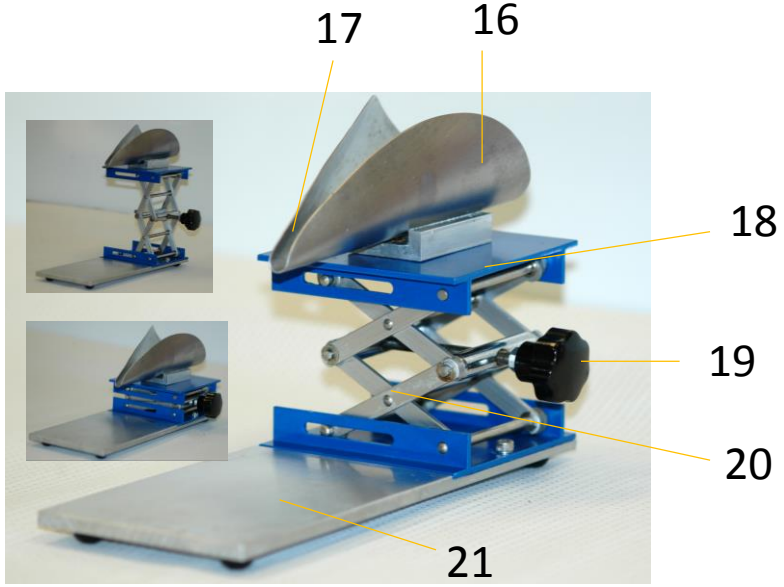
Figure 1.









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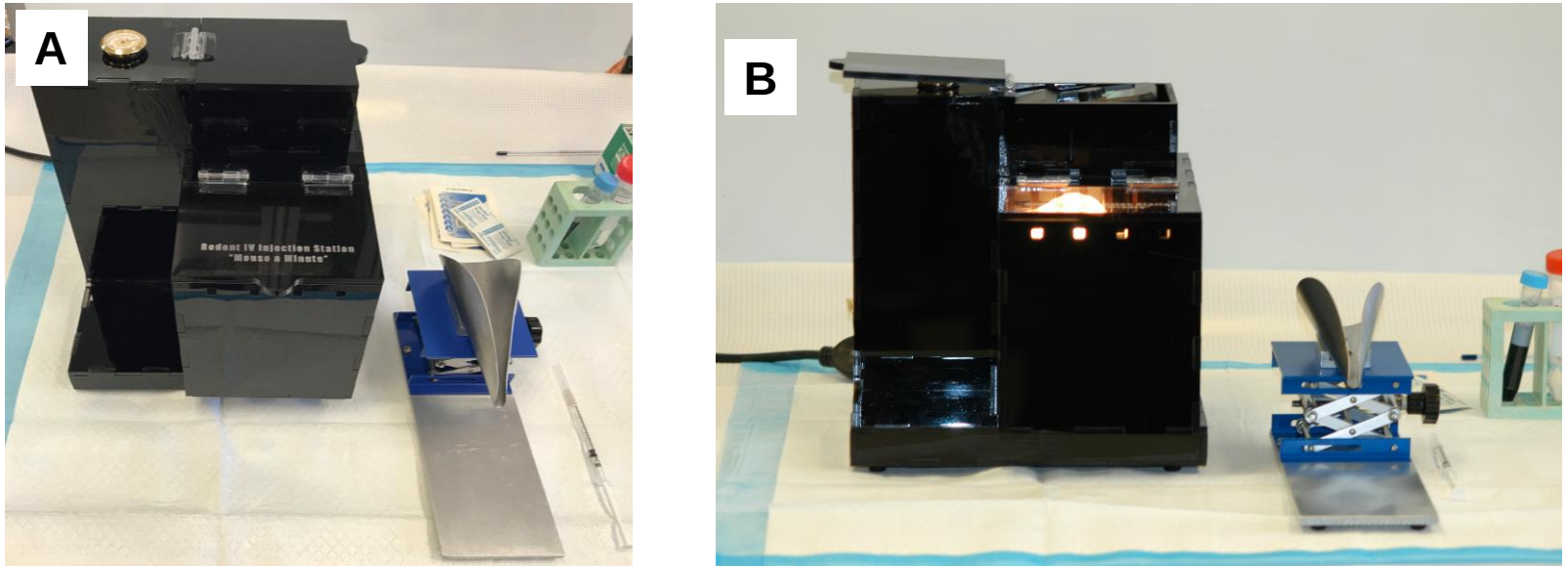

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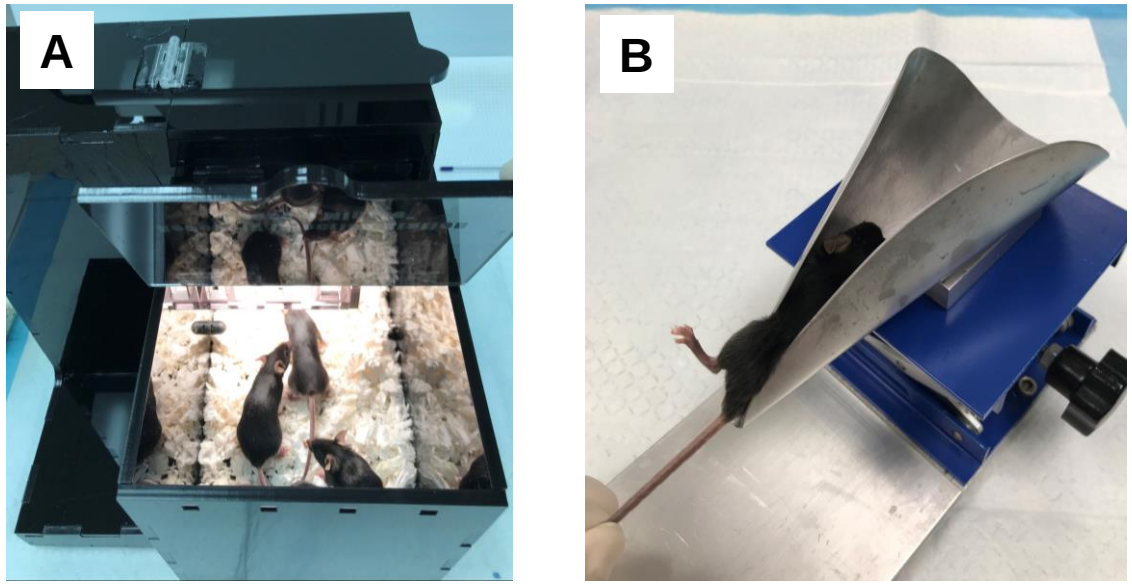

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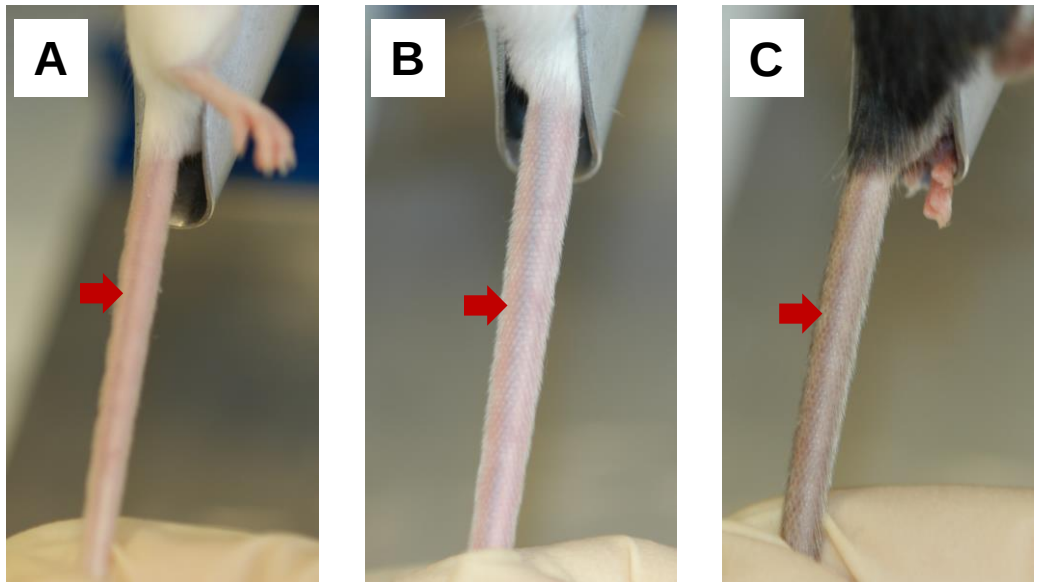

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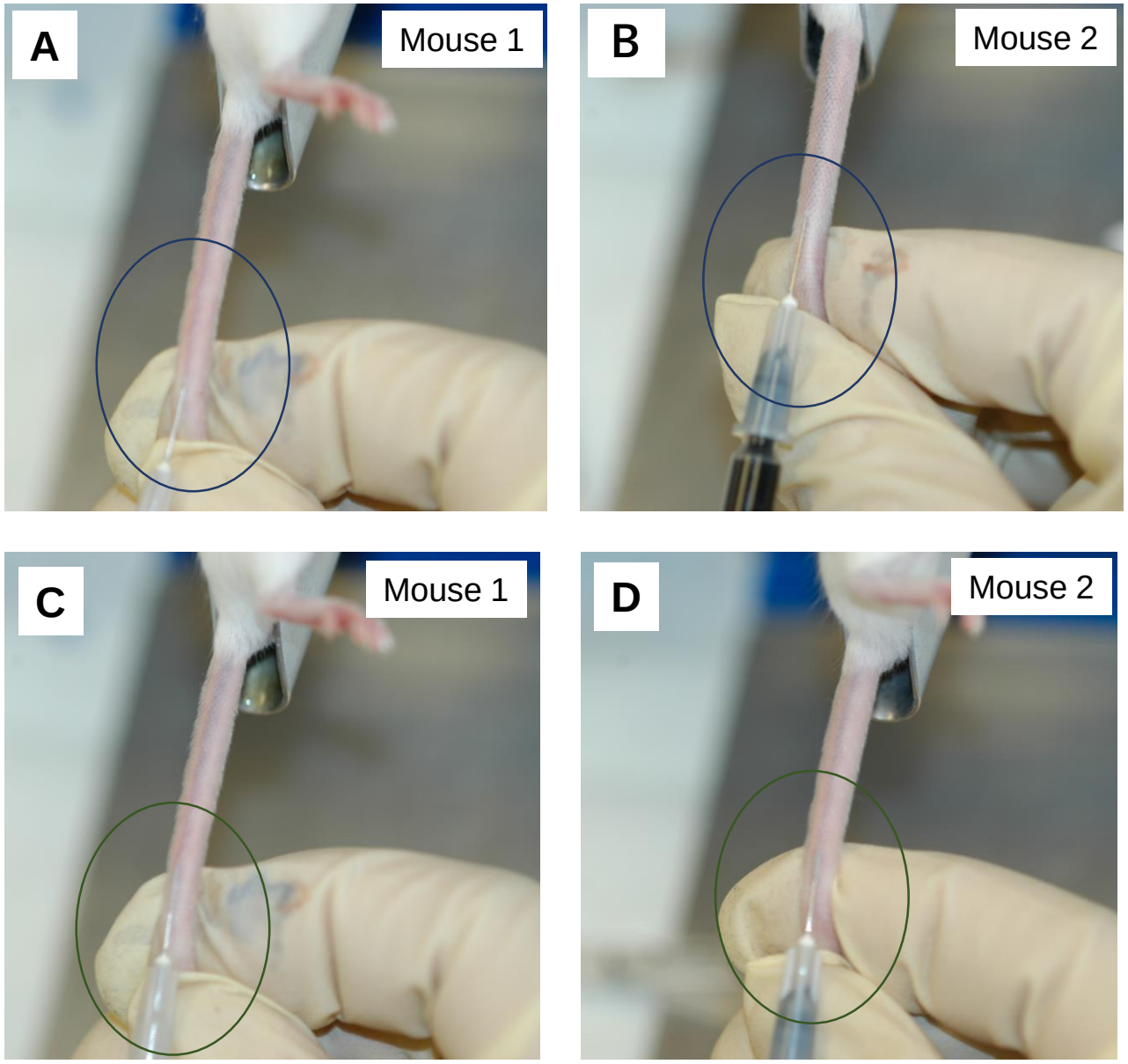

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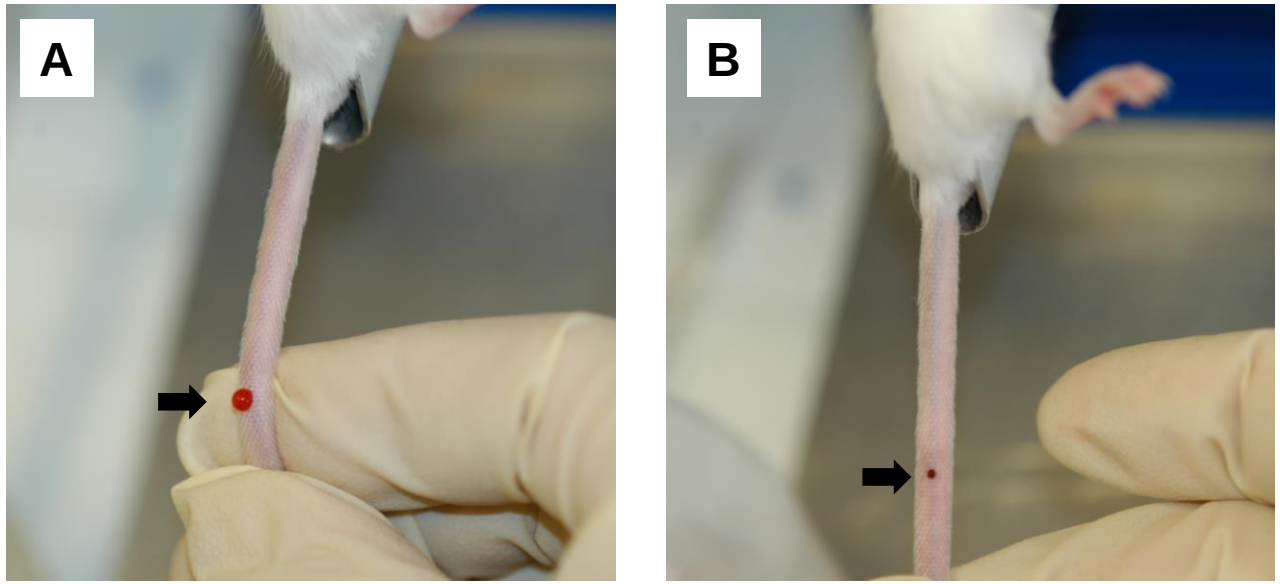

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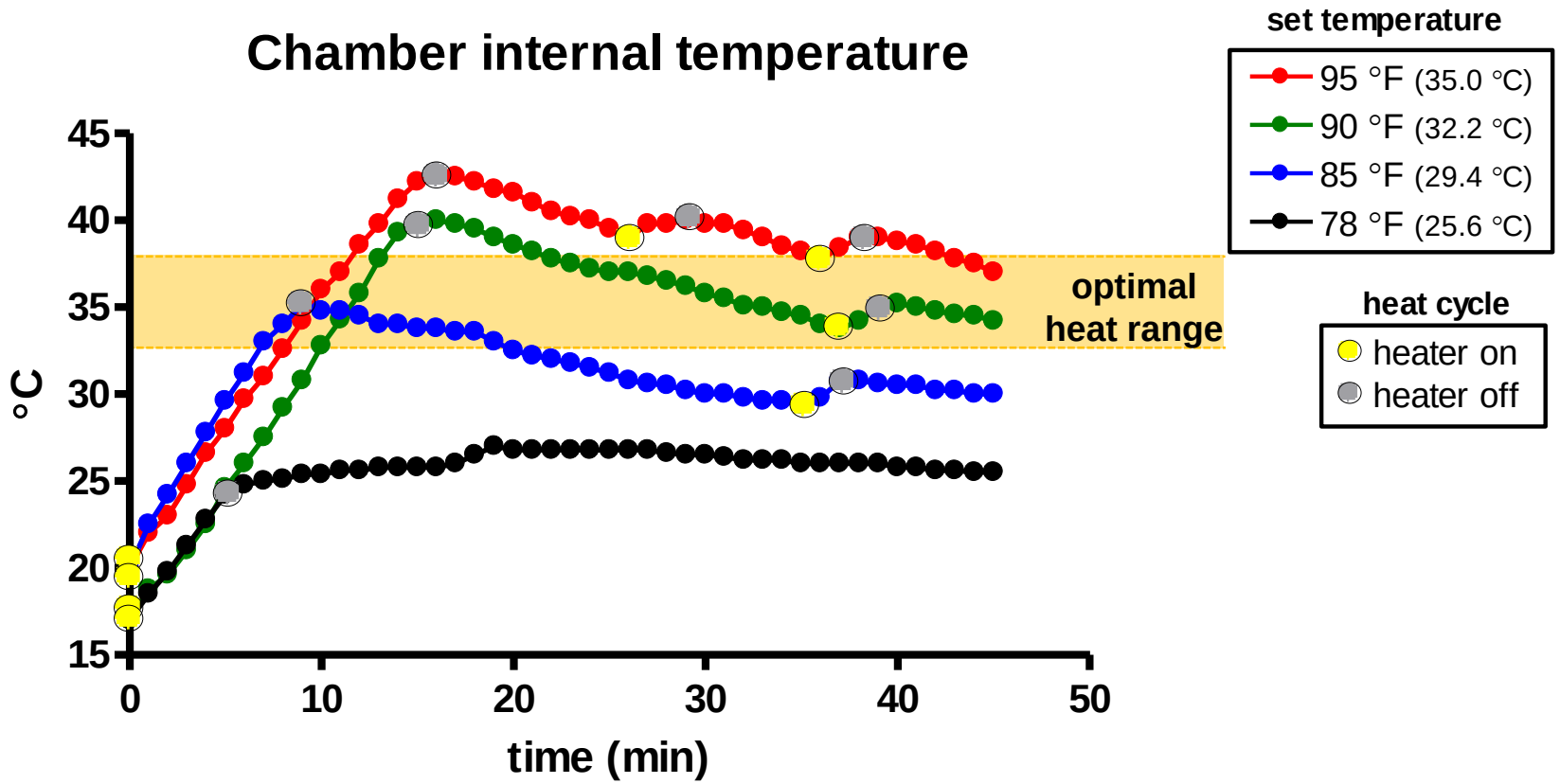
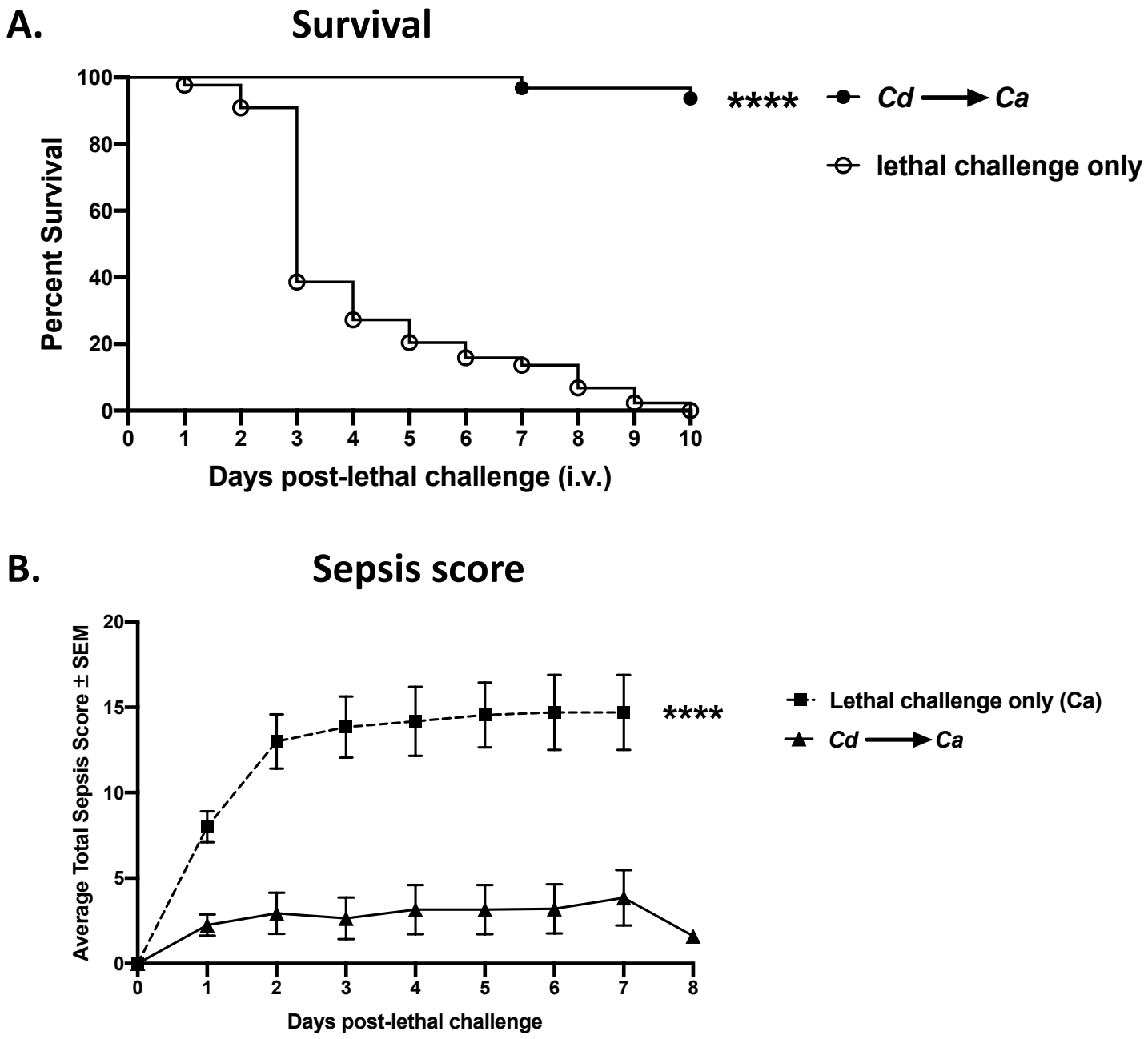

Chamber internal temperature
45
40
35
30
25
20
15
°C
0
10
20
30
40
50
time (min)
optimal
heat range
set temperature
95 °F (35.0 °C)
90 °F (32.2 °C)
85 °F (29.4 °C)
78 °F (25.6 °C)
heat cycle
heater on
heater off

Figure 8.


A.
Survival
Percent Survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
Days post-lethal challenge (i.v.)
****
Cd → Ca
lethal challenge only
B.
Sepsis score
Average Total Sepsis Score ± SEM
20
15
10
5
0
0 1 2 3 4 5 6 7 8
Days post-lethal challenge
****
Lethal challenge only (Ca)
Cd → Ca

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Candida albicans strain DAY185	Carnegie Melon University	N/A	provided by the laboratory of Aaron Mitchell
Candida albicans strain *efg1Δ/Δ cph1Δ/Δ*	University of Tennessee Health Sciences	N/A	provided by the laboratory of Glen Palmer
Candida dubliniensis strain Wü284	Trinity College, Dublin, Ireland	N/A	provided by the laboratory of Gary Moran
Mice	Charles River Laboratories	551NCICr:SW	Female Swiss Webster; 6-8 weeks old
Mice	Charles River Laboratories	556NCIC57BL/6	Female C57BL/6; 6-8 weeks old
Needles, 27G, ½-in	Becton Dickinson	305109	can be substituted from other vendors
Phosphate buffered saline (PBS)	GE	SH30028.02	can be substituted from other vendors
Rodent warming and restraining device (Mouse a Minute)	LSU Health	custom order	Mouse a Minute is available for custom ordering from LSU Health
Sabouraud dextrose agar (SDA)	Becton Dickinson	211584	can be substituted from other vendors
Syringes, 1 mL	Becton Dickinson	309659	can be substituted from other vendors
Trypan blue solution	Sigma	T8154	
Yeast peptone dextrose (YPD) broth	Fisher Scientific	BP2469	can be substituted from other vendors

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript has been proofread. No spelling or grammar mistakes were found.

2. Please make the title concise to directly reflect the protocol and do not include punctuations (colons, etc.).

The title has been revised.

Original title

A protocol for effective and efficient tail vein injections in mice using a contemporary warming/restraining device: a sepsis model example.

Revised title

A protocol for effective and efficient tail vein injections in a murine fungal sepsis model using a contemporary warming/restraining device.

3. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Paragraph indentation has been removed.

4. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Presented here is a protocol …”

The short abstract has been revised within the word limit.

5. The manuscript is the important component of the submission. Please do not refer to the article as video article.

Corrected.

6. Please ensure the Introduction include all of the following with citation:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

The above criteria are addressed in the Introduction.

7. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

The ethics statement has been moved to the beginning of the protocol section before the numbered steps.

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

The numbering of the protocol steps has been corrected.

9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
The protocol section has been revised to use the imperative tense exclusively. Inappropriate phrases have been removed.

10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.
The protocol steps have been revised to shorten the paragraphs.

11. The Protocol should contain only action items that direct the reader to do something.
Sentences unrelated to action items have been removed from the Protocol.

12. Please describe all actions using complete sentences and include all specific details.
The protocol section has been revised to include sufficient details.

13. Please ensure you answer the "how" question, i.e., how is the step performed?
The protocol section has been corrected to better describe the actions.

14. 1.2: What test agents are used in your experiment?
PBS was used as a vehicle in all injections described in Protocol and Representative results.

15. 1.3.1: If this need filming please convert this into action steps and in complete sentence. Please also write the protocol as if you are describing someone how to do it.
The protocol has been revised in the imperative tense. Filming is necessary to illustrate the action steps of the protocol.

16. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Highlighted action steps are now within the 3 pages limit.

17. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
None of the figures presented in the manuscript are previously published.

18. As we are a methods journal, please ensure that the Discussion explicitly cover the following

in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Discussion has been revised to focus on the technical elements and limit non-technical information.

Attached please find an example manuscript for your consideration.

Reviewers' comments:

Reviewer #1:

In this article, Yano et al. describe a protocol for a more efficient method of tail vein injection in a rodent model.
The manuscript is well written and addresses the important scientific issue of variability and efficiency in animal modeling.

My comments are as follows:

1. The author's address a critical issue in small animal modeling, namely wide variability that has plagued the field for years.

2. Authors address this variability in tail vein injection through the novel design of a regulated warming apparatus and adjustable restrainer.

3. 1.2.3. please ensure the symbols reads less than or equal to since 200uL is a common volume used for intravenous mouse modeling.
The less than symbol '<' has been corrected to '≤'.

4. 1.2.5. please include eye protection as part of intravenous injection in the event inoculum splashes into eye mucosal membrane.
Use of safety glasses has been included as part of appropriate PPE for the procedure.

5. 2.2.2. I would recommend providing guidance if signs of hyperthermia are noted. Do the authors recommend that light source is adjusted or turned off and the warming procedure is restarted? It would be valuable to provide this guidance as the authors have the experience with their apparatus.
Animals exhibiting signs of hyperthermia should be returned to their cage and monitored until they resume normal activity prior to reuse. The warming device should be turned off, if not occurring automatically, in case of the internal temperature exceeding the optimal range. This guidance has been added to the protocol

6. 2.3.1. I would recommend specifically noting that lifting mice from base of tail is recommended (per veterinary specialists), not the end of tail to avoid injury or fracture.

This instruction has been added in the section.

7. 3.1 First paragraph. There appears to be a note from the authors about showing difference in tail color, which I would think would be quite useful. You have images available as Supplement Figure 1 - I would combining these images to contrast between lighter and darker colored tails with indicators of the lateral tail vein (as you have done).
The supplemental figure has been moved to Figure 4 to compare the tail skin colors between the two mouse strains.

8. Line 381. Please define "HDC" since it is being used for the first time in the manuscript.
HDC is defined in Introduction, lines 106-107. However, this terminology may not be commonly used outside of the Mycology field. Hematogenously disseminated candidiasis has been spelled out both in Introduction and Discussion.

9. Figure 1 and 2. The description of the device are great, although I would like to ask if the authors intend to offer schematics or the specific components used for construction of this device such that audience/readers will be able to build their own device?
The device is licensed by LSUHSC. Interested parties would need to contact us to get specs, etc. A statement has been added to the end of the Discussion to contact the Corresponding Author if interested in procuring a similar device.

Reviewer #2:
Manuscript Summary:
The authors describe the use of an aperature that provides a heat source and a restraining device that replaces the use of warm water for vasodilation and the help of a second person holding the mouse for the tail vein injection. The use is demonstrated with a systemic Candida albicans infection model. The advantages and disantvantages of the device are discussed comprehensively.

Minor Concerns:
1) Is the light within the heating chamber depending on the bulb that is also the heat source? Or is there a constant/independent light source within the chamber? Turning the lights on/of will likely stress the mice.
The light bulb is the sole heat source of the device controlled by a thermostat. The thermal reflective material of the chamber walls maintains the heat over an extended period of time during which the on/off operation only occurs every 15-20 minutes. Therefore, stress on the animals due to the bulb illumination is minimal.

2) 2.3.4 The treatment with alcohol solution may result in the loss of the achieved vasidilation, especially in C57BL/6 mice. We observed no adverse effects (like inflammation of the injection side) when performing injections without this procedure.
Rodent tails are generally highly cornified, thus the heat loss from brief application of alcohol solution is likely minimal. Gentle cleaning of the tail with wipes or tissues also help remove scales often seen in older mice. Together with providing a more aseptic injection site encourages use of a brief alcohol wipe, but we note that it is not a rigid requirement.

3) 3.1, end of first passage: yes, show the C57BL/6 images and refer to Supplementary Figure 1.
The statement has been corrected and now refer to the C57BL6 images.

4) Figure 8: Since the present manuscript is presenting a method that should provide/improve a high reproducibility, it would be of interest to see outcome of the four different experiments in comparison. This might also be presented as a supplemental figure.
Individual experiments have been included as a supplemental figure.

Reviewer #3:
Manuscript Summary:
The manuscript describes the process of murine tail vein injection, a commonly used technique in murine models of disseminated infection. It focuses on the use of a controlled temperature device and a cone restrainer to facilitate the procedure.

Major Concerns:
1. The technique has already been very well described in a variety of protocol publications, including as a component of multiple JoVE publications. This manuscript has an extreme emphasis on the temperature control device / cone restrainer system that can only be purchased from the author's institution. Cone restrainer systems are widely available and in regular use. There are a variety of temperature regulation devices commercially available. The authors very briefly criticize many commonly used methods without detail as to why this system is better. More importantly, they don't mention that other temperature control devices that are very similar to their device are available from commercial sources.
We agree that there are similar heating or restraining devices available from commercial sources. The manuscript focuses on demonstrating our optimally streamlined protocol and tail vein injection technique by use of the newly designed device. The protocol is unique in that the device is equipped with two pieces of apparatus as one unit that are otherwise obtained separately and may not be effectively compatible with. To our knowledge, such protocols using specific devices are not available in the current literature. Furthermore, an informal needs analysis conducted by survey a few years back suggested such devices are sorely needed. Hence, those using other such sophisticated devices should continue to do so and report accordingly. The protocol we describe is especially useful in experiments requiring tail vein injections to large groups of animals as we describe in the mouse sepsis model.

2. Swiss Webster outbred mice are very rarely used for disseminated candidiasis models. The authors mention that these mice are more susceptible to disseminated candidiasis than are inbred mice. That is a significant concern because the widely used outbred strain for this model (CD-1) is much more resistant to systemic candidiasis than inbred strains.
We agree that use of Swiss Webster mice in disseminated candidiasis models is not common, mainly due to presumed genetic heterogeneity in outbred mice and availability of knockout mice in inbred strains. The issues of variable susceptibility to fungal sepsis between mouse strains are explicitly addressed in Discussion. Evidence suggests that susceptibility to fungal sepsis among outbred strains including Swiss Webster and CD-1 are similar (PMID 29415485, 780278).

Minor Concerns:
1. It is misleading to call Candida spp. the fourth leading cause of hospital acquired bloodstream infection (lines 100-101) because this comparison is made between a group of organisms (Candida spp) versus individual bacterial organisms.
The statement has been revised to clarify specific *Candida* spp. involved in hospital acquired bloodstream infection.

2. What kind of bulb is used in the device? Are replacement bulbs available? with the massive increase in LED style bulbs, will the bulb be available in the future?
The device includes an incandescent light bulb (100 Watts). We are in the process of testing other bulb styles as an alternative heat source.

3. Line 316 includes the text (show BL5 images here?)
The statement has been corrected and now refer to the C57BL6 images.

4. How are "viable" yeast cells enumerated using a hemocytometer? (Line 339)
Viable yeast cells are identified by trypan blue dye exclusion. This procedure has been included in the protocol.

5. A 100 uL inoculum is a relatively low volume. Low volume inoculums are especially susceptible to partial injections
Results are highly reproducible using a 100 µl inoculum volume. The protocol has been revised to indicate that the inoculum volume can be increased up to 200 µl in mice.

6. No detail is provided about how to monitor animals for symptoms, how long to monitor them, and what to do when they are moribund (ie euthanasia is important prior to moribund state).
This information has been included in the protocol.

7. The authors mention that initial temps of 5-7 deg C higher than the set temperature are necessary for loss of heat during transfer. If you lose 5-7 deg during transfer, what happens each time you remove a mouse?
Brief introduction of the ambient air into the warming chamber during mouse removal likely cause a temperature drop at a minimal extent. In this case, the device typically repeats shorter heat cycles to adjust the internal temperature instead of prolonged heating cycles.

8. The y axis in Fig 7 uses deg C but the legend uses deg F
The figure legend has been corrected to reflect the figure labeling.

Reviewer #4:
Manuscript Summary:
The manuscript describes the use of the Mouse-a-Minute heating box and holder for IV injection of mice and uses the systemic Candida albicans infection model as an example in this manuscript, comparing mortality and sepsis progression in immunised and non-immunised (?) mice.
The Mouse-a-Minute device temperature profile and maintenance is described in the manuscript.

This is a well written and very clear manuscript, but there are a few questions regarding the experimental procedures and data that need to be addressed.

Major Concerns:
One major concerns about the manuscript relate to animal welfare and animal handling used. The Mouse-a-minute device used incandescent bulbs as a heat source, but it was not clear if red bulbs were used or bright white bulbs; this could have very different effects on mouse welfare and this should be specified in the manuscript. Please include type of bulb and power/wattage.
The device utilizes a white incandescent bulb with 100 watts output. We have added this information to the protocol. Evidence from several stress models in rodents suggests that intermittent exposures to light during daytime did not significantly affect stress markers (e.g. serum corticosterone levels, activity levels) irrespective of light sources compared to a light exposure during nighttime that resulted in disturbance in their circadian rhythms (PMID 17628617, 25979911). Therefore, we estimate that a level of stress in mice heat-treated with the device is tolerable for tail-vein injections.

Another concern is with regards to animal handling. It is now widely appreciated that picking up mice by their tails is not good handling practice and leads to animals being less easy to handle. In some institutes, picking up by the tail is banned and cupping/tunnel handling used instead. This needs to be addressed in the protocol - there are numerous papers on this - see Gouveia and Hurst Sci Rep 2019 PMID 31889107 as an example.
We agree with the reviewer that lifting rodents by their tails is not a safe handling method in certain circumstances especially in older/heavier mice or pregnant females which are prone to serious injuries with this method. Statements have been included in the revised manuscript to alert users of this issue and to suggest alternative methods of handling when applicable. At any rate we stress that picking them up with the mid-section of the tail is most optimal.

A final concern is the lack of detail for the immunization of the mice prior to infection - there are no experimental details provided for this part of the experiment. Indeed, it is even unclear how many mice were immunized with Candida dubliniensis and how many were immunised with the highly attenuated Candida albicans strain. There are also no details about the Candida dubliniensis strain.
We have revised the Protocol to provide experimental details regarding the immunization designs and specific *Candida* strains used in the protocol. The graphs were actually generated from *C. dubliniensis* but very similar to the *C. albicans* mutant. The graph was meant to serve as a general resource. However, we clarify the *C. dubliniensis* was used and the *C. albicans* mutant shows similar results. Independent experiments for this are shown in the supplemental figure.

Minor Concerns:
Manuscript appears to be adapted from another study which included Staphylococcus aureus. Please remove mention of bacterium and growth medium from the materials table, figure 8B and discussion lines 568-581.
The materials table and Figure 8B have been corrected as suggested. The statements in Discussion regarding intraabdominal and gastrointestinal infection models have been revised accordingly.

Need to add details on the C57BL/6 mice used in some images.
The supplemental figure for tail vein images of C57BL/6 mice has been incorporated in Figure 4. The figure legend has been revised to reflect the change.

From the data shown in Figure 7 it appears that the machine takes 10-15 minutes to reach a stable temperature. Do you advise users to switch on the machine 10-15 minutes before beginning any mouse handling/work? Can this be added to the protocol?
The protocol has been revised to clarify that animals should be placed in the warming chamber when the internal air reaches the preset temperature.

Mouse strain. Is is not clear from the information provided what the weights of the mice are in the Candida albicans infection experiment. The dose seems very low for systemic infection leading to 60% mortality at day . Would be useful if the authors provided references in support of the increased susceptibility of Swiss Webster mice in Candida infection experiments. Something to consider about US Swiss Webster mice... Charles River website says that progeny of their colony were outbred from a single pair to form a new stock - how outbred are these mice? (www.criver.com)
We agree that the origins of mouse strain colonies could have an impact on the variable susceptibility to fungal sepsis. We have included statements to address this variable.

Inoculum Sizes - also need to include references about effects of different inoculum sizes (lines 519-521)
Appropriate references have been included.

Need to include references for appropriate volumes for administering substances in animals e.g. Turner et al JAALAS 2011 PMID 22330705
Appropriate references have been included.

Preparation section
line 132 - remove "proper" from the sentence. It would be assumed that any training provided would be proper - could add that support/supervision would be required until become competent.
We agree and has revised the sentence.

Section 1.2.2 maybe include vehicles used for administration of IV drugs. What is meant by growth medium? Do you mean mammalian cell culture medium or fungal growth medium?
Growth media for mammalian cell culture should be used. We have revised the section to clarify the applicable vehicles for tail vein injection.

Why is some text highlighted?
Highlights indicate the action items for filming for the visualized portion of the manuscript.

Section 2.2
Do you recommend switching on the machine at least 10 minutes before the experiments begins?
Yes we have included this procedure in the protocol.

How was it determined that 20-30 minutes are acceptible/safe as an incubation time? Was this determined experiementally?
Although not formally tested, we have not evidenced any adverse effect based on the typical duration of usage in our laboratory.

Section 2.3.5
Local regulations may determine the maximum number of attempts per animal
We agree and have included a note to consult local IACUC regulations.

Section 3.1
line 314-315 what is meant by "... at modified inocula"?
These are inoculum sizes optimized for specific fungal strains or mouse strains by individual laboratories. We have revised the section to clarify the information.

Line 316 remove comment in brackets and refer to the supplementary figure
This error has been corrected.

Section 3.2
What is specifically meant by "condition the animals"? Do you mean train them in the procedure before the day that you actually carry out the experiment? Or do you mean that you will follow the procedures in Section 2?
This indicate the procedure to induce vasodilation by treating the animals with the rodent warming device per the protocol in Section 2. We have revised the Section 3.2 to clarify this procedure.

Results and Figures
Figure 1 part C - what does 11 indicate? not mentioned in the figure legend for part B
We have revised the figure legend to indicate 11 in both part B and part C.

Figure 1 part D - 20 and 21 might be mis-labelled
This error has been corrected in the Protocol and Figure legend.

Figure 3B mouse looks uncomfortable as if pulled back quite hard - maybe add to legend and description that mouse was pulled back until the base of the tail reached the tip of the cone, then it was gently rotated and the leg positioned upwards.
We believe that this procedure be best demonstrated as a movie and agree that the figure legend need more detailed description how this position can be achieved. The legend has been revised as suggested.

How was 5-10 minute incubation time determined for the device?
We found that heat treatment for a period of 5-10 min is typically needed to induce visible dilation of the tail vein at the recommended temperature range. Since the tail skin is directly exposed to air without fur, the incubation time largely depends on the room temperature prior to heat treatment.

Figure 4C does not add to the figure (not much more detail that 4B) - could be removed. Legend:

for part A don't need "that courses" in the sentence - could be very confusing to a non-native English speaker.
We have made the changes in Figure 4 and the legend.

Figure 7 Graph's main axis is in degree centigrade but data legend is in Farenheit (centrigrade in brackets) - explain why this is (machine thermometers are not centigrade!)
We recognize that Centigrade should be used consistently. Although the device thermostat is scaled in Fahrenheit as fabricated in the US, we indicate all Fahrenheit values in Centigrade in the manuscript. We are in the process of resolving this issue with the production team to fabricate all subsequent devices to utilize Centigrade exclusively.

Figure 8 and description of results. The figure legend does not describe what the data in part B represent and how many mice they represent (no of mice; mean/median; standard deviations/SEMs). How many mice were immunized with Candida dubliniesis? How many were immunized with the highly attenuated Candida albicans strain? Should they be amalgamated into a single dataset? Are the data in the graph data amalgamated from 4 experiments or a representative set? Needs to be clear in the figure legend and should be clear how many mice are used in each group and in each graph.
Need to include details of the vaccination protocols also state now non-immunized mice were treated.

In figure 8, there is relatively tight data until day 4... then the day of death becomes much more variable. Is this due to injection variation or inoculum effects or variation between the different experiments.
We have included a supplementary figure to show 4 independent experiments with *C. dubliniensis*. The higher variability seen at later time points appear to be due to the variations between animals (e.g. varying microbial burden and severity in tissue damage) which occurred reproducibly in all 4 separate experiments.

Discussion of C. albicans strains (line 530) where mention CAF-2 and CAI-4; these are all derivatives of SC4314 (not independent wild type strains).
This statement has been removed.

There is some disconnect in the manuscript regarding male and female mouse susceptibility to infection - in some places it says that there is a difference, then in another place it says that there is no difference. Need to support by references. Could depend on whether inoculum is adjusted to take into account weight differences.
We agree and have removed the statements in discussion regarding sex differences in Swiss Webster mice. Although literature suggests that differences in susceptibility to an i.v. challenge between Swiss Webster males vs. females are minimal, it is possible that the effect of sex differences may be more pronounced in inbred strains.

Need to change Balb/c to BALB/c throughout
This error has been corrected.

Supplemental Figure

Supplemental figure 1.