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Infection of mice with Citrobacter rodentium ICC180 by natural transmission

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Abstract

Citrobacter rodentium is a Gram-negative bacterium which infects laboratory mice in a similar way to how enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) infect humans. We routinely use a bioluminescent derivative of *C. rodentium* called ICC180 which allows us to monitor infection dynamics non-invasively using biophotonic imaging.

Experimental infection is typically initiated via oral gavage but we have previously shown that *C. rodentium* rapidly spreads between infected and uninfected animals and that bacteria shed from infected mice are 1000 times more infectious than laboratory-grown bacteria with a different tissue tropism.[1,2] In this protocol, we describe the different methods for establishing an infection using natural transmission.

1. Wiles, S., Dougan, G., & Frankel, G. (2005). Emergence of a 'hyperinfectious' bacterial state after passage of *Citrobacter rodentium* through the host gastrointestinal tract. Cellular microbiology, 7(8), 1163–1172. https://doi.org/10.1111/j.1462-5822.2005.00544.x
2. Bishop, A. L., Wiles, S., Dougan, G., & Frankel, G. (2007). Cell attachment properties and infectivity of host-adapted and environmentally adapted Citrobacter rodentium.*Microbes and infection*,*9*(11), 1316–1324. https://doi.org/10.1016/j.micinf.2007.06.006

Guidelines

Experiments involving animals and pathogenic bacteria require ethical and biological safety approval. When planning experiments involving animals, consult the **PREPARE** and **ARRIVE** guidelines.

Materials

Microorganism: *Citrobacter rodentium* ICC180

Growth media and chemicals:

	Item	Catalogue number	Supplier
	LB (Lennox) Agar	240110	Fort Richard Laboratories
	LB (Lennox) Broth	240230	Fort Richard Laboratories
	Kanamycin	K4000	Sigma-Aldrich
	Phosphate Buffered Saline tablets	P4417	Sigma-Aldrich
	Isoflurane		MedSource NZ Ltd.
	Glycerol	1040922511	Merck

Plasticware and equipment:

	Item	Catalogue Number	Supplier
	Petridishes - 90mm x 14mm	S9001	medi'Ray NZ
	Falcon 50mL Conical Centrifuge Tube	BDAA352070	In vitro technologies
	1.7 mL microcentrifuge tube	AXYGMCT175C	Global Science

	Item	Catalogue Number	Supplier
	straight 4 cm Instech stainless steel feeding needle		Harvard Apparatus
	Pipette tips		
	Pipettes - various sizes		
	37 degree incubators - shaking and static		
	Vortex		
	Centrifuge		

Animals and husbandry:

6–7 week old C57BL/6Elite mice from specific-pathogen-free (SPF) stocks. We routinely work with female mice as male mice have a tendency to fight and this has an impact on infection dynamics, with stressed animals experiencing a more severe infection.

	Item	Supplier
	Tecniplast Blue line 1284L cages	Tecniplast Australia Ltd
	Grit-ology 1/8″ corn cob	Corn-cob-ology, Mt Kuring gai NSW, Australia
	EnviroDri	Biological Associates, Gladesville NSW, Australia
	mouse house	Tecniplast Australia Ltd

	Item	Supplier
	Teklad global 18% protein	Biological Associates, Gladesville NSW, Australia

We house up to 6 animals in individually HEPA-filtered Tecniplast Blue line 1284L cages with sterile bedding materials (Grit-ology 1/8″ corn cob and EnviroDri), a mouse house, and autoclaved cardboard tube for enrichment. We provide the animals with free access to sterile food (Teklad global 18% protein) and water. Conditions in the animal unit are controlled at 20-24°C, 45-65% relative humidity, and a 12-hour dark-light cycle. Lights turn on at 6:30 am and off at 6:30 pm with a 30 min dawn/dusk period starting at 6 am and 6 pm, respectively.

Troubleshooting

Before start

Prior to oral gavage, ensure animals have been weighed and marked in some way so that you can identify individual animals. We weigh animals by placing them in a 1ml pipette tip box (with the insert removed) placed on a set of scales. We use a marker pen to mark each animal's tail. For example, if there are 5 animals in a cage, we give each animal 1-5 lines across their tails. In our experience, a black marker pen stays visible the longest.

Preparation of *Citrobacter rodentium* ICC180

2d 0h 5m

1 At least two days before needed, revive bacteria from frozen stocks stored at –70-80°C. Plate onto LB-Lennox media. At this stage, you can grow them with or without kanamycin [M] 50 ug/mL . Incubate Overnight at 37 °C

1d

2 The day before needed, inoculate 10 mL LB-Lennox (LB) media supplemented with kanamycin in a 50 mL tube. We generally inoculate using several colonies to provide a more heterogeneous culture for infection. Incubate Overnight at 37 °C with shaking at 200 rpm .

1d

3 On the day of infection, centrifuge the culture at 4500 rpm for 00:05:00 and resuspend in 1 mL PBS to give a 10x concentrated inoculum.

5m

4 To retrospectively calculate the number of bacteria in the inoculum, prepare a 10-fold dilution series of the inoculum in PBS and incubate 3 25 µL drops of each dilution onto LB plates (with or without kanamycin). Incubate overnight at 37 °C and count the colonies.

Oral gavage of seed mice

5 [Optional] Animals can be lightly anaesthetised using gaseous isoflurane to aid gavage. To do this, place mice into the anaesthetic induction chamber and induce anaesthesia using a flow rate of 1 L/min oxygen combined with 5% isoflurane. Animals are sufficiently anaesthetised once the animals have lost their righting reflex.

It is important that animals are not too deeply anaesthetised as their vital functions can be compromised. The respiratory rate of a normal undisturbed mouse is approximately 180 breaths per minute. A slow rate drop of 50% is acceptable during anaesthesia. Breathing should be steady. If the animals' breathing becomes ''jerky'', too much anaesthetic is being applied and this will be fatal if maintained for long periods of time. If an animal appears too deeply anaesthetised, immediately turn off the anaesthetic and administer supplemental oxygen.

6 Transfer the inoculum to a 1 mL syringe and attach a feeding needle.

7 Using the feeding needle, orally gavage each animal with 200 µL of concentrated inoculum.

This video is a good resource for people who are new to the technique: **https://researchanimaltraining.com/articles/oral-gavage-in-the-mouse/**.

To minimise the risk of oesophageal trauma and incorrect dosing, it is crucial that the operator is skilled both in the technique and the restraint method used. Inadvertent dosing into the lung may occur, and this usually results in the animal showing immediate signs of respiratory distress. If this is observed, then the animal should be humanely killed using an approved method.

8 After dosing, return animals to their cage and observe. If done correctly, the animals should resume normal activity within minutes.

9 Animals should be routinely monitored by measuring their weight, behaviour, and condition. The GRIMACE scale is ideal. The original study that developed the scale is online **here** and an explanatory poster and other resources are available **here**.

Depending on the size of the dose, some animals may not eat for a short period and so may experience some weight loss in the first 24 hours after gavage. If they are active and alert and their fur remains smooth and glossy, this is usually no cause for concern.

Natural transmission of *C. rodentium* from seed to naive mice

10 At the peak of infection (usually between days 6-10 post-gavage) move one or more infected mice to a clean cage.

11 Add naive animals to the clean cage alongside the infected animals. *C. rodentium* will transmit via coprophagia and grooming. Transmission usually occurs within 48 hours.

12 All animals should be routinely monitored by measuring their weight, behaviour, and condition. The GRIMACE scale is ideal. The original study that developed the scale is online **here** and an explanatory poster and other resources are available **here**.

Natural transmission of *C. rodentium* from the environment

13 [Optional] Instead of cohousing naive and infected mice together, infection can be initiated from a contaminated environment. At the peak of infection (usually between days 6-10 post-gavage), transfer infected animals to a clean cage.

14 After 18-48 hours, remove infected animals from the cage and remove some stool from the cage into a preweighed microcentrifuge tube. This will enable you to retrospectively calculate the level of environmental *C. rodentium* contamination.

15 Add naive animals to the dirty cage. *C. rodentium* will transmit via coprophagia and generally occurs within 48 hours.

16 To retrospectively calculate the level of environmental *C. rodentium* contamination, add PBS to a final concentration of 0.1 g of stool per 1 mL of PBS. Vortex briefly, then prepare a 10-fold dilution series in PBS and incubate 3 25 µL drops of each dilution onto LB plates supplemented with [M] 50 ug/mL kanamycin. Incubate overnight at 37 °C and count the colonies.

Establishing a transmission chain

1d

17 [Optional] To establish a defined transmission chain, co-house one infected mouse (usually around the peak of infection) and one naive mouse together in a clean cage. *C. rodentium* should transmit via coprophagia and grooming within 48 hours.

18 Around the peak of infection, collect stool samples from infected mice to monitor infection levels and reinitiate the transmission chain should natural transmission fail.

18.1 Label and weigh individual microfuge tubes. Write the weight on the side of the tube. Remove inserts from pipette tip boxes

18.2 Place the infected mouse in a clean pipette tip box. Once the mouse has produced 1-3 individual stools, transfer these to a sterile labelled microfuge tube of known weight using a pair of clean tweezers.

Depending on the mouse strain, stools can become very loose and much more difficult to pick up using tweezers. In these instances, try using a small laboratory spatula instead. We've also observed mice placing their stools up on the walls of the tip box like they are moving them out of the way or making an offering.

18.3 Transfer the mouse back to its home cage and clean the pipette tip box.

18.4 Weigh the tube again to find the weight of the stool.

18.5 Add PBS to a final concentration of 0.1 g of stool per 1 mL of PBS. Homogenise using a vortex.

18.6 To retrospectively determine the number of bacteria present, prepare a 10-fold dilution series in PBS and incubate 3 25 µL drops of each dilution onto LB plates supplemented with [M] 50 ug/mL kanamycin. Incubate overnight at 37 °C and count the colonies.

18.7 To the remainder of the stool homogenate, add an equal volume of [M] 50 % (v/v) glycerol to give a final concentration of [M] 25 % (v/v) and store at -70 °C or below.

19 If transmission fails to occur, re-established the transmission chain using an inoculum derived from the stool homogenate frozen in glycerol. Use a sample of the stool homogenate to inoculate 10 mL LB-Lennox (LB) media supplemented with kanamycin in a 50 mL tube. Incubate Overnight at 37 °C with shaking at 200 rpm and oral gavage as previously described.

1d