



Published in final edited form as:

Methods Mol Biol. 2012 ; 921: 131–142. doi:10.1007/978-1-62703-005-2_18.

Animal Models of *Helicobacter*-Induced Disease: Methods to Successfully Infect the Mouse

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Abstract

Animal models of microbial diseases in humans are an essential component for determining fulfillment of Koch's postulates and determining how the organism causes disease, host response(s), disease prevention, and treatment. In the case of *Helicobacter pylori*, establishing an animal model to fulfill Koch's postulates initially proved so challenging that out of frustration a human volunteer undertook an experiment to become infected with *H. pylori* and to monitor disease progression in order to determine if it did cause gastritis. For the discovery of the organism and his fulfillment of Koch's postulates he and a colleague were awarded the Nobel Prize in Medicine. After *H. pylori* was established as a gastric pathogen, it took several years before a model was developed in mice, opening the study of the organism and its pathogenicity to the general scientific community. However, while the model is widely utilized, there are a number of difficulties that can arise and need to be overcome. The purpose of this chapter is to raise awareness regarding the problems, and to offer reliable protocols for successfully establishing the *H. pylori* mouse model.

Keywords

H. pylori mouse model; Mouse orogastric inoculation; *H. pylori* culture; *H. pylori*-specific PCR; Helicobacter genus PCR; Microaerobic; *H. pylori* selective media

1. Introduction

With the discovery of *Helicobacter pylori* and its association with gastric disease in humans (1), the search for an animal model to study the organism and its pathogenesis became of great importance. Even more interest in an animal model was generated when *H. pylori* was classified as a Class 1 carcinogen by the World Health Organization (2). A mouse model was the most logical approach from a cost and housing consideration. However, it soon became apparent that it is very difficult to persistently colonize immuno-competent mice with *H. pylori* (3–5). Short-term colonization (2–4 weeks at most) was reported by a few laboratories, but this was not promising with respect to development and study of chronic gastritis and peptic ulcer. It also made treatment and immunization studies difficult. Other animal models described were gnotobiotic pigs (6), conventional piglets (7), primates (8), cats (9), germ-free mice (10), nude mice (11), gnotobiotic beagle dogs (12), and Mongolian gerbils (13) which are the only wild-type rodent species that develop gastric carcinoma following *H. pylori* infection without other tumor-promoting factors (14).

A promising mouse model was reported by Lee et al. (15) using *Helicobacter felis* which was originally isolated from the stomach of cats (16). A close relative of *H. pylori*, this organism colonizes mouse stomachs long term and can produce low-grade B-cell gastric

lymphomas indistinguishable from those in infected humans (17). Because of the ease of colonization and similarity to human disease, this model became widely used. The ferret was found to be naturally infected with *H. mustelae* which causes chronic gastritis (18) and was also initially explored as a potential model for human *H. pylori* infection (19). However, while these models generated data that was highly relevant to *H. pylori* in humans, they used organisms that are similar to *H. pylori*, but they are not *H. pylori*. It was not until Lee et al. isolated the Sydney strain of *H. pylori* and showed it could colonize mice long term that the mouse model using *H. pylori* became viable for a wide range of research laboratories (20). Using this mouse-adapted strain, good levels of colonization were maintained in conventional C57BL/6 and Balbc mice. After 8 months, chronic active gastritis developed which progressed to severe atrophy. This laboratory also assessed the number of organisms needed to colonize mice and also looked at the susceptibility of different strains of mice to colonization (21). They found that C57BL/6 > Balbc > Swiss Webster mice were colonized with *H. pylori* in that order with C57BL/6 mice exhibiting the best colonization. In 2004, a second mouse-adapted *H. pylori* isolate with virulence factors differing from *H. pylori* Sydney was described by Thompson et al. to serve as a model for determining the importance of various virulence factors in the progression of disease (22). Another strain of *H. pylori* reported to cause disease in the mouse model is the B128 strain (23, 24). Table 1 lists some of the conventional strains of mice susceptible to *H. pylori* and *H. felis* colonization. Rogers and Houghton have written a review describing the mouse model in enterohepatic helicobacter-induced carcinogenesis, including *H. pylori*, and include a description of genetically engineered mice susceptible to development of cancer (25).

The *H. pylori* Sydney strain remains the organism of choice for use as a model of human disease. However, this model can be very difficult to reproduce in the laboratory due to a number of factors including culture viability, inoculum, frequency of dosing, repeated passage of the organism, and mouse strain chosen. The protocols detailed below address these problems and provide guidance aimed at repeated successful colonization.

2. Materials Needed

2.1. Media

1. Autoclave.
2. Pipetman and pipet tips.
3. 1.5 ml Cryovials.
4. Brucella broth/agar.
5. Trypticase soy agar plates.
6. Blood Agar Base.
7. Bacitracin.
8. Amphotericin B.
9. Vancomycin.
10. Polymyxin B.
11. Naladixic Acid.
12. Sterile standard petri dishes (100 × 20 mm).
13. Defibrinated Horse blood.
14. Brucella broth with 20% glycerol.

15. Urea agar slants.
16. Fetal bovine serum.
17. Freeze media: 80 ml of double-distilled water to the amount of brucella broth powder needed for 100 ml total volume, and then adding 20 ml of glycerol. This is referred to as freezing medium; freezing media.

2.2. Culture

1. 37°C Incubator with rotating platform.
2. BD GasPak system vented jars (Becton Dickinson, cat no. 260627).
3. Anaerobic gas mix (80:10:10/N₂:CO₂:H₂).
4. Tubing (VWR red vacuum tubing, cat. No. 62995-059).
5. Clamps.
6. T fittings.
7. Vacuum pump (capable of vacuum down to 20 in. of mercury).
8. 37°C Incubator.
9. Sterile swabs.
10. Spectrophotometer (visible capability).
11. Phase contrast microscope.
12. Light microscope.
13. Gram's stain reagents.
14. 250 ml Flasks with screw caps or sliding metal caps.
15. Biosafety hood.

2.3. Mouse Inoculation

1. 1 cc Syringes.
2. 24 gauge × 1 in. stainless steel feeding tubes.

2.4. PCR

1. Thermocycler.
2. Bovine serum albumin.
3. dNTPs.
4. Taq polymerase.
5. Primers: C97 (5'-GCT ATG ACGGGT ATCC) and C05 (5'-ACT TCA CCC CAG TCG CTG). Hp1: AAA GCT TTT AGG GGT GTT AGG GGT TT and Hp2:AAG CTT ACT TTC TAA CAC TAA CGC (26).
6. PCR Buffer.
7. Sterile distilled water.
8. Eppendorf tubes.
9. Agarose.

10. Gel box.
11. Electrophoresis power supply.
12. Ethidium bromide.
Dilute a sample of the stock solution to 0.5 µg/ml with water. Protect from light.
13. Documentation center for taking photos of gels.
14. 1 kb Plus DNA Ladder.
15. TAE Buffer.

3. Methods

3.1. Microbial Stock Solutions

Excessive passage of the isolates is known to affect colonization and virulence, so it is necessary to freeze several vials of inoculum to last for about 6 months. The number of stock vials that you keep will depend on the number of experiments you anticipate.

1. One dram vials, or equivalent, are filled with 1 ml of brucella broth containing 20% glycerol.
2. The vials are then autoclaved and stored at room temperature.
3. Stock is prepared from *H. pylori* grown on agar plates (trypticase soy blood agar) for 2–3 days. Do not use cultures that are older as they will yield poor, if any, growth on plating.
4. Growth is harvested from the plates using a sterile swab dipped in freezing medium and the swab swirled in a sterile, cool vial. The medium in the vial should then be lightly turbid. Several vials can be prepared from one plate.
5. These vials are then frozen at –80°C. Freezing at –20°C is not recommended, nor is refreezing thawed vials of stock as the organism will not be viable (see Note 1).

3.2. Growth of *H. pylori* for Mouse Inoculation

All cultures are incubated in a microaerobic environment. *H. pylori* can be grown on blood agar plates or in broth for mouse inoculation. (see Note 2).

3.2.1. Selective Media

1. The antibiotics in Table 2 are added to autoclaved and cooled Blood Agar Base (Sigma Chemical Company) along with 5% sterile horse blood and after gentle but thorough mixing to avoid bubbles, poured into standard sized petri dishes.
2. This media can be kept at 4°C for up to 2 months if stored in plastic containers to keep the plates moist.
3. Always do quality assurance using a known *H. pylori* to ensure good growth.

¹Replace frozen stock at least every 6 months with a fresh isolate from an experimental animal with good gross and histologic lesions. This is important because *H. pylori* has been known to lose its ability to colonize on prolonged storage and also on multiple passages on laboratory media.

²It may seem like a lot of work to prepare your own media, and *H. pylori* will grow on other selective media, but many other gastric organisms compete, especially lactobacilli, making it difficult to identify the *H. pylori*. Almost nothing else will grow on the selective media, making life a lot easier in the long run.

3.2.2. Blood Agar Plates

1. Either commercially bought or in-house prepared plates may be used but the plates should be fresh and moist.
2. Trypticase soy agar, brain–heart infusion agar, brucella agar, or Blood Agar Base can be used but all must be supplemented with 5% whole blood or fetal calf serum.
3. Serum can be used in place of whole blood as red blood cells are not necessary for good growth. However, the use of blood agar makes the *H. pylori* colonies more easily visible since they have a watery appearance when confluent that often looks like “no growth” to the novice. Either horse blood or sheep blood can be used. Blood may be stored frozen and thawed for making plates if in-house plates are prepared. Lysis of the blood does not affect the growth of *H. pylori* though, again, colony visibility can be an issue on translucent plates, especially if quantitative cultures are performed. An added advantage of using agar plates is that you can visually detect contamination.
4. Plates should be inoculated using a sterile swab dipped into a thawed stock vial to yield a heavy, confluent growth after 2–3 days of microaerobic incubation.
5. Approximately 2 ml of inoculum can be obtained from each plate.
6. Growth is harvested using a sterile swab moistened in brucella broth. After swiping the plate several times, the organisms are harvested by swirling the swab in brucella broth. The plate can be swiped a second time to get as much of the growth as possible.
7. At the same time as preparing this inoculum, inoculate new plates with the swab and incubate to use for your second inoculation of the mice in a day or two (see Notes 3 and ⁴).
8. When all plates have been harvested, the motility and morphology of the organisms should be observed using a phase contrast microscope. While there will be more coccoid forms (round balls instead of gently curving spirals) and less motility in cultures from agar plates as compared to broth, it is important that the majority of organisms (roughly 80% or more) have good morphology and are motile. If the culture is too old, coccoid forms will predominate and these will not colonize mice.
9. The optical density is then measured at a wavelength of 660 nm. The optical density should be between 0.8 and 1.4 to ensure enough viable organisms for good colonization. As mice receive 3 doses of organism, repeat this procedure a total of three times in order to prepare fresh inoculum for each of the 3 doses. At the end of 3rd harvest session, all the remaining cultures are discarded and the process from thawing stock to preparation of inoculum started totally anew for subsequent experiments.

3.2.3. Broth Culture

1. Broth culture is more difficult because contamination is not readily apparent and the likely hood for contamination is increased by the manipulations of the flask. However, morphology and motility are exceptional in a young broth culture. Five hundred milliliter flasks with screw caps work well, though flasks with metal sliding caps can also be used.

³Always prepare your next inoculation plates/flasks first before you do any other manipulation of the culture in order to minimize the risk of contamination.

⁴Always use Gram’s stain broth cultures and agar plate cultures to ensure a pure culture.

2. Each flask is filled with 150 ml of brucella broth and then autoclaved. On cooling, 7.5 ml (5%) of fetal calf serum is added.
3. Flasks are inoculated with an *H. pylori* suspension prepared from freshly grown agar plates inoculated from stock as described above. It is not recommended to inoculate the flask directly from frozen stock because the lag time for the organism to grow well can take several days. Each flask should be inoculated with 1 ml of suspension.
4. Sliding metal caps allow for good gas exchange with the culture, but be careful that screw caps are left loose.
5. The flasks are then placed in jars that can be vented. Two-sided tape on the bottom of the jar prevents the flask from sliding during shaking.
6. After venting the jars to obtain a microaerobic environment, the jars are incubated at 37°C overnight with shaking on an orbital shaker at 100 rpm.
7. When turbidity is observed, and this may be on day 2, using phase microscopy, assess the morphology and motility. There should be minimal (<10%) coccoid forms and good motility.
8. New flasks are inoculated with 1 ml of the culture from the turbid flasks and then incubated as before for the next mouse inoculation (see Note 1).
9. The broth is then centrifuged at 8,450×g for 20 min, and the supernatant discarded.
10. The pellet is suspended in 10 ml of Brucella broth and the optical density at 660 nm determined. Adjust the optical density to between 0.8 and 1.4 for your inoculum using brucella broth.

3.2.4. Obtaining a Microaerobic Environment

1. All agar plates and flasks are placed in jars with lids that enable a good seal and a vent allowing for evacuation of the jar and filling with gas mixture. An example of this type of jar is the BBL vented jars used with the Gas-Pak system. Use of an anaerobic mix (80:10:10/N₂:CO₂:H₂ or 90:5:5/N₂:CO₂:H₂) in a vented jar system works best.
2. Use a vacuum pump connected by tubing through a T fitting to tubing to a gas gauge. The tubing from the third port of the T is connected to another T fitting that is connected to the gas tank and the third port is for attaching to the jar.
3. A clamp is placed between the vacuum pump and the first T fitting (Fig. 1). Using tubing on the vent of the jar lid, connect the vent on the jar to the unused port of the second T fitting and make sure that the clamp is not engaged.
4. Turning on the vacuum pump, evacuate the jar down to 20 in. of mercury as indicated by the gas gauge.
5. Use the clamp to close the connection to the pump. Turn off the vacuum pump. The vacuum in the jar should hold at 20 in. If it does not, then the jar is not sealed and you need to use another, or determine why it is leaking. Once you have determined that the jar is sealed, slowly fill the jar with the gas mix until the gas gauge reads 0. If you fill too rapidly, you will blow fungi, etc. into the flasks/plates.
6. Clamp off the tubing on the jar vent and incubate as directed above (see Note 5).

3.3. Inoculation of the Mice

Only personnel who are appropriately trained and approved by the institution's Committee on Animal Care should orally gavage mice. This is a very exacting procedure and esophageal damage and pulmonary aspiration and/or tracheal damage can result in the immediate or prolonged death of the mouse.

1. For *H. pylori* inoculation, each mouse receives 0.2 cc of inoculum intragastrically using a 24 gauge \times 1 in. straight stainless steel feeding tube attached to a 1 cc syringe. This is done without anesthesia. This results in an inoculum of roughly 2×10^8 organisms. The mice will receive a total of 3 doses, 1 per day and each separated by 1–2 days.

3.4. Verification of Colonization

1. Two to 3 weeks post inoculation, if verification of colonization is required one or two mice should be euthanized and the gastric tissue assessed by culture and/or PCR for *H. pylori* (see Note 3).
2. Intestinal shedding of other enterohepatic *Helicobacter* spp. allows PCR of fecal samples to determine colonization, thus sparing the need to euthanize an animal.
3. Shedding of *H. pylori* in feces is not a reliable indicator for colonization. If PCR is positive for *H. pylori*, then colonization is confirmed. A negative PCR does not mean that mice are not colonized.

3.4.1. Culture of *H. pylori* from Gastric Tissue (see Note 4)

1. A small piece (approximately 1 or 2 mm square) of antrum or body tissue should be collected into a sterile vial (similar to a 1 dram vial) containing 0.5–1 cc of freezing medium and kept on ice until processed.
2. The sample can be frozen at -80°C , or processed immediately.
3. To process, use a sterile glass tissue grinder (3 ml capacity), pour the tissue and broth into the tissue grinder tube, and homogenize with the glass pestle until the suspension is homogeneous. Disposable tissue grinders are not recommended for gastric tissue as the tissue is too difficult to homogenize.
4. Plate 0.1 cc of the homogenized tissue onto a small section of selective media (below) and streak to obtain isolated colonies.
5. Incubate the plate microaerobically for 3–5 days, checking at 3 days and 5 days if necessary. Individual colonies are small (1 mm or less) and water droplet in appearance (see Note 5).
6. To ensure that these are *H. pylori*, a rapid urease test (below) should be performed and a Gram's stain examined for typical morphology.

3.4.2. PCR for *H. pylori*

1. Tissue for PCR should not be placed into brucella broth with glycerol as the glycerol interferes with the PCR. Collect these samples in an empty vial.
2. These can be processed immediately or frozen at -20°C . This temperature is sufficient as the DNA is to be analyzed and viability is not an issue.

⁵A microaerobic environment can also be achieved using BBL-type jars and Campy Paks (or similar) or anaerobic paks without catalyst, but it is not known if all *H. pylori* isolates will grow well using this system. Many *Helicobacter* spp. grow best in the presence of hydrogen. Using the GasPak system does, however, negate the need for gas tanks, vacuum pumps, and vented jars.

3. Tissue is homogenized in 200 μ l of sterile PBS using sterile disposable plastic tissue grinders. These are acceptable for PCR samples because they have not been exposed to helicobacter-infected tissues previously, and the resultant homogenate, though not homogeneous, will be further digested with the reagents in the kit.
4. Extract using the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Indianapolis, Ind.) using the tissue extraction protocol.
5. *Helicobacter* genus-specific primers C97 and C05 are used to amplify a 1.2-kb PCR product from the 16SrRNA gene. These primers are excellent for detecting *Helicobacter* species and will be reliable only if mice are not colonized with other *Helicobacter* spp.
6. Ten microliters of the DNA preparation is used for a PCR 100 μ l reaction mix.
7. The PCR mixture contains 10 μ l Taq polymerase buffer, 0.5 mM each of the two primers, 200 mM each deoxynucleotide, and 200 mg of bovine serum albumin per ml and 0.53 μ l Taq polymerase.
8. Amplification conditions are as follows: initial denaturation at 94°C for 5 min, then 94°C for 1 min, annealing at 58°C for 2 min, and elongation at 72°C for 3 min for a total of 35 cycles completed before a final elongation step at 72°C for 8 min.
9. A 15 μ l aliquot of the PCR product is electrophoresed through a 1% agarose gel separation matrix prior to ethidium bromide staining and viewing under a UV light.
10. If other *Helicobacter* spp. are present in the mice, *H. pylori*-specific primers HP1, Hp2 can be used to verify colonization. They are used in the same proportions and cycling conditions described above for the genus primers.

3.4.3. Rapid Urease Test—Using a straight wire inoculating needle, pick an individual colony and stab the needle into a urea agar slant.

1. For a positive test, after no more than 2–3 min, the stab should be turning a bright pink.

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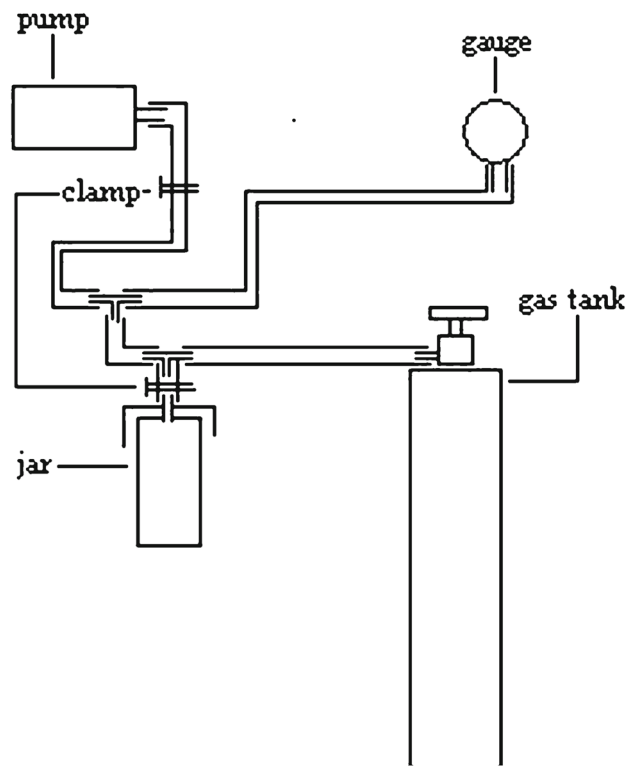


Fig. 1.
Venting system for microaerobic culture conditions.

Table 1

Mouse susceptibility to *H. pylori* and *H. felis* challenge

Mouse strain	Colonization by <i>H. pylori</i> SS1	Pathology	Colonization by <i>H. felis</i>	Pathology
C57BL/6	Yes	Chronic active gastritis progressing to severe hyperplasia and atrophy	Yes	Chronic active gastritis progressing to severe hyperplasia and atrophy
Balbc	Yes	Chronic active gastritis progressing to severe atrophy	Yes	Some gastric atrophy, less apoptosis, mild antral gastritis, MALToma
SJL	Yes, but low numbers	Mild gastritis	Yes	Corpus gastritis
C3H/He	Yes, but low numbers	Moderate to severe chronic active gastritis	Yes	Corpus gastritis
DBA/2	Yes, but low numbers		Yes	Corpus gastritis
CBA	Yes, but low numbers	Mild gastritis	Yes	Mild antral gastritis
Swiss Webster	Yes, but low numbers	Mild gastritis	Yes	Moderate self-limiting gastritis

Table 2

Antibiotics for selective media preparation

Antibiotic	Final concentration	Sigma catalogue number
Amphotericin B, solubilized	50 µg/ml	A 9528 50 mg (~45% Amph. B)
Vancomycin HCl	100 µg/ml	V 2002 1 g
Polymyxin B sulfate	3.3 µg/ml	P 1004 5,000,000 units (~7,870 U/mg)
Bacitracin	200 µg/ml	B 0125 50,000 units
Naladixic acid, Na salt	10.7 µg/ml	N 4382 1 g