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TITLE:

Mouse Models of *Helicobacter* Infection and Gastric Pathologies

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Helicobacter pylori, *Helicobacter felis*, mouse model, MALT lymphoma, inflammation

SUMMARY:

Mice represent an invaluable *in vivo* model to study infection and diseases caused by gastrointestinal microorganisms. Here, we describe the methods used to study bacterial colonization and histopathological changes in mouse models of *Helicobacter pylori*-related disease.

LONG ABSTRACT:

Helicobacter pylori is a gastric pathogen that is present in half of the global population and is a significant cause of morbidity and mortality in humans. Several mouse models of gastric *Helicobacter* infection have been developed to study the molecular and cellular mechanisms whereby *H. pylori* bacteria colonize the stomach of human hosts and cause disease. Herein, we describe protocols to: 1) prepare bacterial suspensions for the *in vivo* infection of mice via intragastric gavage; 2) determine bacterial colonization levels in mouse gastric tissues, by polymerase chain reaction (PCR) and viable counting; and 3) assess pathological changes, by histology. To establish *Helicobacter* infection in mice, specific pathogen-free (SPF) animals are first inoculated with suspensions (containing ≥ 10^5 colony-forming units, CFUs) of mouse-colonizing strains of either *Helicobacter pylori* or other gastric *Helicobacter* spp. from animals, such as *Helicobacter felis.* At the appropriate time-points post-infection, stomachs are excised and dissected sagittally into two equal tissue fragments, each comprising the antrum and body regions. One of these fragments is then used for either viable counting or DNA extraction, while

the other is subjected to histological processing. Bacterial colonization and histopathological changes in the stomach may be assessed routinely in gastric tissue sections stained with Warthin-Starry, Giemsa or Haematoxylin and Eosin (H&E) stains, as appropriate. Additional immunological analyses may also be undertaken by immunohistochemistry or immunofluorescence on mouse gastric tissue sections. The protocols described below are specifically designed to enable the assessment in mice of gastric pathologies resembling those in human-related *H. pylori* diseases, including inflammation, gland atrophy and lymphoid follicle formation. The inoculum preparation and intragastric gavage protocols may also be adapted to study the pathogenesis of other enteric human pathogens that colonize mice, such as *Salmonella* Typhimurium or *Citrobacter rodentium*.

INTRODUCTION:

Helicobacter pylori is a spiral-shaped, Gram-negative, human gastric pathogen present in all populations across the world, with infection rates in developing countries estimated to be in the order of 80%[1]. Although most *H. pylori*-infected individuals are asymptomatic, some develop more severe diseases, ranging from peptic ulceration to gastric cancer[2]. *H. pylori*-associated cancers are broadly characterized either by malignant changes in epithelial cells (GECs) or by the formation of extra-nodal lymphoid tissues in the stomach, resulting in gastric adenocarcinoma or mucosa-associated lymphoid tissue (MALT) lymphoma, respectively. *H. pylori* is highly adapted to survive in the harsh ecological niche of the stomach due to the presence of various virulence factors and mechanisms facilitating its adherence, growth and metabolism in this niche. In particular, virulent strains of *H. pylori* possess the 40 kb *cag* Pathogenicity Island (*cag*PAI) that encodes 30 genes required for the production of a Type 4 secretion system (T4SS)[3,4]. *cag*PAI-positive *H. pylori* strains are associated with the induction of higher levels of chronic inflammation in the host, which interestingly has been implicated as an essential precursor of gastric adenocarcinoma[5].

In vivo animal models, particularly mice, have been highly informative by allowing researchers to investigate the relative contributions of host, bacterial and environmental factors on *H. pylori* infection and disease outcome[6]. Studies have previously demonstrated that prolonged *H. pylori* infection of mice on the C57BL/6 genetic background results in the development of chronic gastritis and gland atrophy, both hallmarks of *H. pylori* infection[7]. Furthermore, infection with the related feline/canine bacterial species, *H. felis*, has been shown to induce MALT formation in mice with similar pathology and disease progression as seen in human MALT lymphoma[8,9]. The most commonly used *H. pylori* isolate in mouse colonization studies is the "Sydney Strain 1" (SS1) strain[10], which is *cag*PAI$^+$ but has a non-functional T4SS (T4SS$^-$)[11]. Other widely used strains include *H. pylori* B128 (*cag*PAI$^+$/T4SS$^+$)[12] and X47-2AL (*cag*PAI$^-$/T4SS$^-$)[13]. For *H. felis* infections, the strain CS1 ("Cat Spiral 1", *cag*PAI$^-$/T4SS$^-$) is generally used[14].

Herein, we provide a protocol describing the preparation of *Helicobacter* inocula for *in vivo* infection, the procedure for intragastric gavage of mice, as well as methods for the processing of tissues for the study of histopathological changes in the stomach. In particular, this article will focus on the histological methods used to visualize bacterial colonization and assess histopathological changes, including MALT formation, in the gastric mucosa of infected mice.

Some of the methods described here may be adapted to the study of other gut pathogens such as *S.* Typhimurium or *C. rodentium*.

PROTOCOL:

1. Growth and Preparation of Bacterial Inocula

1.1. Thaw glycerol stocks of *H. pylori* or *H. felis*[15] from -80 °C and subculture on horse blood agar (HBA) plates comprising: Blood Agar Base No.2 (see **Table of Materials**); a modified "Skirrow's antibiotic selective supplement" (consisting of vancomycin, 10 µg/mL; polymyxin B, 25 ng/mL; trimethoprim, 5 µg/mL; amphotericin B, 2.5 µg/mL); and 5-10% (v/v) horse blood[15,16]. The bacteria grow well under microaerobic conditions in 2.5 or 3.5 L anaerobic jars containing the appropriate gas packs (see **Table of Materials**), at 37 ˚C.

Note: *H. pylori* strains grown under these conditions must be subcultured after 1-1.5 days of incubation, whereas for *H. felis*, at least 2 days of incubation is generally required. Suitable culture and storage media have been described in detail previously[15].

1.2. Prepare bacterial inocula for mouse infection from early–to-mid logarithmic phase cultures. Harvest bacteria gently from agar plates by flooding each plate with 1-2 mL of brain heart infusion (BHI) broth. Aspirate suspensions from plates with Pasteur pipettes.

1.2.1. Alternatively, prepare inocula from *Helicobacter* bacteria that have been propagated in BHI broth for 16-18 h[15]. In this case, collect bacteria by low speed centrifugation at 2,200 x g for 10 min at 4 °C.

1.3. Assess the viability and motility of the bacteria by examining wet mount preparations under phase contrast microscopy (100X objective). Prepare wet mounts by resuspending a loopful of bacteria in a 10-20 µL droplet of BHI broth on a glass microscope slide. In the case of *H. felis*, which is a much larger bacterium than *H. pylori*, use a haemocytometer to accurately count the numbers of viable bacteria. Confirm culture purity by performing a Gram-stain.

Note: Only use *H. pylori* inocula if the majority of bacteria have a bacillary or spiral shape (the morphology can vary depending on the strain). *H. felis* inocula should primarily contain helical-shaped bacteria. Do not use inocula if most bacteria have a coccoid morphology, as these forms are not viable and will not establish an infection in mice.

1.4. Estimate the number of bacteria in the inoculum by counting under phase contrast microscopy the approximate number of bacteria per field (100X objective) and by using the following guide: 1 bacterium per field = approximately 10^6 colony-forming units (CFU) of *Helicobacter*/mL; 10 bacteria per field = approximately 10^7 CFU/mL; 100 bacteria per field = approximately 10^8 CFU/mL, *etc.*

1.4.1. When using a haemocytometer, calculate CFU/mL using the following formula: CFU/mL = (average number of bacteria in a 4 x 4 field) x (dilution factor) x (10^4).

1.5. Adjust the bacterial cell density of the inoculum to approximately 10^7-10^8 CFU/mL by dilution in BHI broth, if necessary.

1.5.1. To ensure maximal bacterial viability, use inocula for intragastric gavage as soon as possible after preparation.

1.5.2. Always confirm *H. pylori* cell density and viability by performing viable counting of inocula immediately after the gavage procedure (see below). This is not always possible for *H. felis*, as it does not usually form isolated colonies on culture media. The numbers of viable *Helicobacter*s in inocula cannot be determined by optical density measurement (A_{600}) alone as this method does not discriminate between viable (*i.e.*, bacillary/spiral/helical) and non-viable (*i.e.,* coccoid) bacteria.

1.5.3. Use optical density values as a means of estimating the numbers of viable *H. pylori* bacteria in inocula, but in this case, it is first necessary to generate a growth curve. For this, the A_{600} values of *H. pylori* cultures are monitored over time and correlated directly against the numbers of viable bacteria, determined by plate counting.

Note: A convenient method for performing such growth curve determinations is to culture bacteria in liquid medium (Section 1.2), using standard flat bottom tissue culture flasks placed in a 10% CO_2 incubator. The numbers of CFUs, determined from aliquots of the cultures obtained every 4-6 h over 2-3 days, are then compared to the corresponding A_{600} values[17]. Importantly, growth curves must be generated for each *H. pylori* strain, as these may grow at different rates and, furthermore, not all strains grow well in 10% CO_2.

2. Intragastric Gavage of Mice with *Helicobacter*

Note: This method of intragastric gavage can be applied to other bacterial species that colonize the gut *e. g. S.* Typhimurium*, C. rodentium*, *Listeria monocytogenes*.

2.1 Use 6-8-week-old, specific pathogen-free (SPF) and *Helicobacter*-free male or female mice. Use animals with a C57BL/6 genetic background for infection experiments with *H. pylori* or *H. felis*. In the present study, use wild-type (WT) and genetically modified C57BL/6 mice lacking a key innate immune receptor (termed knock-out or KO animals).

Note: Mice on other genetic backgrounds can also be used for *Helicobacter* infections, however, colonization levels and disease severity may be impacted by the type of host background[18,19].

2.2 Aspirate the bacterial inoculum (Step 1.5) into disposable 1 mL syringes and replace the supplied needles with 23 gauge needles onto which are affixed disposable polyethylene

catheters (length, 6-8 cm; internal diameter, 0.58 mm). Fasten catheters to the needles by the application of small strips of plastic film (see **Table of Materials**). Alternatively, replace the needle/catheter assembly by using sterile plastic feeding tubes (20 gauge x 38 mm).

2.3 Physically restrain mice with a firm grip at the scruff of the neck and tail.

Note: This procedure can be performed without anaesthesia, or alternatively, with the use of an inhaled anaesthetic, such as methoxyflurane or isoflurane[15].

2.4 Insert catheter into the center of the open jaw and guide in a caudal direction towards the esophagus. Extend the neck of the mouse to allow ease of access to the stomach through the esophagus (and away from the trachea) until most or all of the catheter is no longer visible and a resistance is felt, corresponding to the base of the stomach. Deliver a specific aliquot, usually 100 µL per inoculation (**Figure 1**).

Note: Mice should be gavaged with ≥ 10^5 CFU to ensure optimal colonization and disease pathology.

2.5 House mice in an SPF animal facility for the duration of the experiment.

Note: Severe pathology and adenocarcinoma in WT C57BL/6 mice is only observed at approximately 24 months' post-infection[20]. However, this effect may be accelerated in some genetically modified animals, or in mice with other genetic backgrounds.

2.6 Upon completion of the gavage procedure, perform a modification of the Miles and Misra technique to determine the numbers of viable *H. pylori* bacteria administered to mice. For this, the inoculum is serially diluted (from 10^{-1}-10^{-5}) in BHI using the method described in detail previously[15].

Note: In order to ensure isolation of single colonies, HBA plates should be warmed and dried in a biological safety cabinet or 37 °C incubator for 10-15 min prior to use.

3. Harvesting Tissues from Mice Post-Experiment

3.1 Euthanize mice by either carbon dioxide inhalation or cervical dislocation, according to the relevant ethics committee for animal experimentation.

3.2 Open the abdominal cavity and excise the stomach using fine, curved scissors.

Note: Sera can also be collected by cardiac puncture to aid in the investigation of systemic responses to *Helicobacter* infection. Additionally, the collection of spleens and paragastric lymph nodes are useful in studying adaptive immune responses.

3.3 Cut the stomach along the greater curvature and remove residual food by gentle washing in sterile phosphate buffered saline (PBS) in a 50 mL tube.

3.4 Wash the stomach again in sterile PBS and then record the wet weight using tared 6 cm plastic Petri dishes.

3.5 Flatten the stomach and dissect sagittally into two equal tissue fragments, each comprising: the antrum, body and non-glandular forestomach regions (**Figure 2**). Remove the non-glandular region and weigh one half of each stomach before adding to either 1 mL of sterile BHI (for viable counting) or snap freezing in liquid nitrogen (for DNA/RNA extraction).

Note: Tubes containing tissue in BHI must be stored on ice until they are ready to be processed. Snap frozen stomach tissues can also be used to extract RNA or proteins for qPCR (quantitative PCR) or western blotting analyses, respectively.

3.6 Add the other stomach half to a 15 mL tube containing 10% formalin. Immerse tissues in 10% formalin for 10 s and then flatten to the top side of tubes. Allow tissues to fix before re-immersing in 10% formalin solution for a minimum of 24 h.

Note: Tissues can remain in 10% formalin for many weeks prior to processing for histology. Prolonged storage of tissue may, however, affect its architecture and/or antigenicity resulting in sub-optimal results in downstream analyses.

4. Confirmation of Bacterial Colonization in the Stomach Post-Infection

4.1 *Viable Counting of H. pylori in the Stomach*

4.1.1 Supplement sterile HBA plates with additional antibiotics (200 µg/mL bacitracin and 10 µg/mL naladixic acid) prior to performing colony counts from infected mouse stomachs[16].

4.1.2 Homogenize stomach sections either manually, using autoclavable polypropylene micropestles, or using a mechanical dissociation instrument (see **Table of Materials**).

4.1.3 Prepare duplicate serial dilutions (10^{-1}-10^{-2}) of the resulting gastric homogenates in sterile BHI.

Note: Dilutions should be decided based on the typical bacterial loads obtained for a given *H. pylori* strain used for infection, as well as the duration of infection. Undiluted samples can also be used.

4.1.4 Divide pre-dried HBA plates (see Note above) into three or four segments. Using an adaptation of the Miles and Misra technique, add 10-100 µL of each dilution onto a segment of the agar plate and spread using sterile plastic loops[15].

4.1.5 Allow the plates to dry and then place them in an inverted position (lid side down) in anaerobe gas jars. To maintain humidity in the jars, include a Petri dish containing water.

4.1.6 Incubate jars at 37 °C until colonies form (typically 4–7 days).

4.1.7 Enumerate segment(s) containing between 10 and 100 isolated colonies.

Note: *H. pylori* colonies and *H. felis* growth on plates can be distinguished from those of other members of the murine gastric microbiota using standard urease, catalase and oxidase tests. *H. pylori* and *H. felis* are positive for all three tests.

4.1.8 Calculate the bacterial loads as (CFU/g of tissue), using the following formula: [(Average number of colonies counted) × (dilution factor) x (volume plated)]/(stomach weight).

4.2 *Detection of H. felis Infection in Gastric Tissues by the Polymerase Chain Reaction (PCR)*

4.2.1 Extract DNA from mouse stomachs using standard DNA isolation protocols, or a commercially available kit.

4.2.2 Determine the DNA concentration of samples using a fluorometric quantitation technique (see **Table of Materials**).

4.2.3 Set up PCR reactions targeting a 325-base pairs (bp) fragment of the *H. felis* urease B gene (*ureB*)[21]. Each reaction should contain: 100 ng of genomic DNA; 1 μM each of forward (5'-AAA ATC CAC GAA GAC TGG GG-3') and reverse (5'-CTT TTA TCC AAG TGG TGG CAC ACC-3') primers; 200 μM dNTPs; 0.5 units of Taq Polymerase and the appropriate amounts of buffer and nuclease-free water.

Note: This oligonucleotide pair has been designed to recognize and bind to homologous sequences in both *H. pylori* and *H. felis ureB* genes but when subjected to the PCR conditions below, not those present in the *ureB* genes of enterohepatic *Helicobacter* spp.

4.2.4 Perform PCR amplification using the following thermal profile: heating at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 61 °C for 30 s and 72 °C for 1 min, before holding at 20 °C.

4.2.5 Run PCR products on a 2% agarose gel for 30 min at 100 V.

5. Histological Analyses of *Helicobacter*-Infected Mouse Stomach Sections

5.1 *Processing of Stomach Tissues*

5.1.1 Remove stomach tissues from formalin and place in clean Petri dishes.

5.1.2 Cut stomach tissues with a scalpel into several equal-sized longitudinal strips (each 2-3 mm thick) and place in labelled embedding cassettes containing foam padding.

5.1.3 Fix stomach tissues by placing embedding cassettes in a jar filled with 80% ethanol.

Note: Stomachs can be processed immediately or stored for up to 1-2 days prior to proceeding to the next step.

5.1.4 Process stomachs on an automated tissue processor, programmed with the following settings:
Dehydration: 70% ethanol - 1 cycle, 20 min; 90% ethanol - 1 cycle, 20 min; 100% ethanol - 2 cycles, 20 min each + 1 cycle, 40 min + 1 cycle, 1 h.
Clearing: xylene – 2 cycles, 30 min each + 1 cycle, 45 min.
Impregnation: paraffin wax at 60 °C - 1 cycle, 45 min + 1 cycle, 1 h + 1 cycle, 1.25 h.

5.1.5 Remove the processed samples from the machine and store at room temperature for paraffin embedding.

5.2 Paraffin Embedding of Processed Gastric Tissues

5.2.1 Place stainless steel base molds on the stage of the embedding unit to warm the bases of the molds.

Note: Embedding machine should be set at 60 °C for efficient paraffin embedding.

5.2.2 Place waxed cassettes containing the samples into a warm wax bath/hot plate area of the embedding unit until wax fully dissolves.

Note: It is recommended that the sample be embedded shortly after wax dissolves. This ensures that hardening of tissues does not occur.

5.2.3 Fill half of the stainless-steel molds with paraffin wax. Using warm forceps, remove stomach tissue strips from the cassettes and gently push the stomach strips through the paraffin to the base of the molds. Carefully orientate tissue strips at a right angle to the base of the molds so that their sliced ends are facing upwards.

Note: The following steps should be performed as quickly as possible to avoid hardening and separation of the paraffin layers within embedded blocks. The proper orientation of stomach tissues is critical for downstream analyses.

5.2.4 Place molds on the cold plate of the embedding unit to fix the specimens in place and re-orientate tissues if necessary.

Note: If the tissues become dislodged and the paraffin begins to harden, place molds back onto the hot plate to melt the wax and re-embed tissues in molds.

5.2.5 Place half of the labelled embedding cassettes (which were used for the tissue processing) on to the top of the molds and gently fill with warm wax. Do not allow paraffin to overflow.

5.2.6 Gently place molds on to a cold plate and allow to cool.

5.2.7 Once the paraffin has fully set, separate the embedded blocks from molds. Clean excess paraffin wax on cassette edges using a hot plate (set above 80 °C) or a scraper. Blocks can be stored at room temperature until sectioning is performed.

5.3 Sectioning of Tissues

5.3.1 Chill paraffin-embedded tissue blocks on ice and heat a water bath filled with ultrapure water to 40-45 °C.

5.3.2 Secure the blade in the holder of the microtome and set the clearance angle between 1°-5° to prevent contact between the block face and knife facet, before inserting paraffin blocks.

Note: Ensure blocks are clear of excess paraffin acquired from the embedding, as this may hinder the fit of the block.

5.3.3 Orientate the blade for a straight cut across the block. Gently cut 2-3 thin sections to ensure correct positioning of the block.

5.3.4 Trim blocks by a thickness of approximately 10-30 μm. This step ensures that a maximal surface area of each tissue strip will be cut.

5.3.5 Cut 10 μm sections and discard any that contain holes caused by trimming.

5.3.6 Carefully pick up sections using tweezers and float them in the water bath for flattening. Use tweezers to separate each section.

5.3.7 Collect sections from the water bath and place onto charged glass slides (see **Table of Materials**).

5.3.8 Store slides upright in a slide rack and place in an incubator at 37 °C. Dry sections overnight.

5.3.9 Store sections at room temperature indefinitely for subsequent analyses.

5.4 Haematoxylin and Eosin (H&E) Staining of Stomach Tissues

5.4.1 Dewax slides using 3 washes of xylene for 5 min each, followed by 3 washes in 100% ethanol, for 3 min each. Ensure fresh solutions are used at each stage.

5.4.2 Rinse slides in tap water for 30-60 s.

5.4.3 Remove excess water by gently tapping the bottom of the slides on a paper towel. Stain with filtered Haematoxylin for 3 min. Ensure that sections are sufficiently covered with the solution.

5.4.4 Rinse slides under running tap water until water runs clear.

5.4.5 Dip slides in Scott's Tap water for 8-10 s. Do not expose slides to the solution for over 10 s as this will result in darker and intense stains. Staining of sections can be viewed under a microscope. Efficient staining at this stage will result in a 'baby blue' color.

Note: Prepare Scott's Tap water by dissolving 2 g of sodium hydrogen carbonate ($NaHCO_3$) and 20 g of magnesium sulphate ($MgSO_4$) in 1 L of distilled water.

5.4.6 Rinse slides in tap water for 30-60 s.

5.4.7 Remove excess water as described in step 3, and then stain with filtered 1% aqueous Eosin for 3 min.

5.4.8 Rinse slides under running tap water until water runs clear.

Note: Staining can be assessed using a light microscope. If staining is too dark, slides can be dehydrated in 100% ethanol for longer than the specified time. If darker staining is required, slides can be stained with Eosin for 1-2 minutes longer, prior to proceeding to subsequent steps.

5.4.9 Dehydrate slides using 3 washes of 100% ethanol for 30 s each, followed by 3 washes of xylene for 2 min each. Ensure fresh solutions are used at each stage.

5.4.10 Mount slides with mounting medium. Add a drop of mounting medium in the center of a clean coverslip prior to gently placing slide on top with sections facing downwards.

Note: Do not dry slides prior to cover slipping.

5.4.11 Place slides on a flat surface and allow to air dry. Slides can also be dried in a fume hood to accelerate drying time.

5.5 Giemsa Staining of Stomach Tissues

5.5.1 Dewax slides using 2 washes of histolene for 5 min each, followed by 2 washes of 100% ethanol for 3 min each and then a final wash in 70% ethanol for 3 min. Ensure fresh solutions are used for each wash.

5.5.2 Rinse slides in tap water for 30-60 s.

5.5.3 Prepare Giemsa solution by mixing 20% Giemsa stain with 80% distilled water. Stain slides with Giemsa solution for 1 h.

5.5.4 Place slides in 100 mL of distilled water containing 3-4 drops of acetic acid for 2-3 s.

Note: Solution must be mixed well prior to use. At this stage, slides should appear pale blue in color.

5.5.5 Wash slides in 96% ethanol for 30 s.

5.5.6 Wash slides in 3 baths of isopropanol for 2 min each, followed by 3 baths of histolene for 2 min each. Use fresh isopropanol and histolene for each wash.

5.5.7 Coverslip slides with mounting medium, as described earlier.

REPRESENTATIVE RESULTS:

This protocol describes an oral gavage technique to achieve intragastric infection with *H. pylori* or *H. felis* in murine mouse models (**Figure 1**). Following euthanasia, stomachs are removed, weighed and divided into 2 equal halves comprising the antrum, body and non-glandular regions of gastric tissues (**Figure 2**). The non-glandular region is removed prior to performing any analyses.

Successful colonization of animals is typically confirmed by performing viable counting on *H. pylori*-infected gastric homogenates, and subsequently enumerating individual colonies on HBA plates (**Figure 3**). Alternatively, PCR is employed to verify infection with *H. felis* using specific, validated primers directed at a 325-bp region of the *H. felis* and *H. pylori ureB* genes (**Figure 4**).

Gastric tissues are processed, embedded and sectioned for downstream histological applications. The H&E staining technique is used to assess the histopathology in *Helicobacter*-infected mice. In the current example, WT C57BL/6 mice display moderate signs of inflammation, including hyperplasia (enlarged mucosa) and gland atrophy at 6 months' post-infection with *H. felis*. The presence of cellular infiltrates can also be observed in the sub-mucosa. Interestingly, however, more severe inflammation is observed in KO mice at the same time point, with the additional presence of lymphoid follicles located in close proximity to cellular infiltrates (**Figure 5**). Finally, *H. felis* bacteria are observed in Giemsa-stained sections of infected mouse stomachs (**Figure 6**).

FIGURE LEGENDS:

Figure 1: Image demonstrating the oral gavage technique. A disposable 1 mL syringe and flexible catheter are used to deliver ≥ 10^5 CFU of bacterial inocula to a mouse via the intragastric route. The mouse was anesthetized using methoxyflurane and held in a firm grip at the neck, allowing for access of the catheter to the stomach via the esophagus.

Figure 2: Harvesting of mouse spleens and stomachs post-infection. Mouse stomachs were harvested post-euthanasia and their contents removed by scraping with a scalpel and washing in sterile PBS. The tissues were then weighed and flattened on a cotton sheet to reveal 2 equal halves, each comprising the gastric antrum, body and non-glandular regions; Scale bar = 10 mm.

Figure 3: Viable counts on *H. pylori*-infected mouse stomachs. Mice on the C57BL/6 background were inoculated with 10^7 CFU of *H. pylori* SS1 and left for 8 weeks. (A) Dilutions of each gastric homogenate are plated onto a half (or third) of an HBA plate and bacterial loads assessed by enumerating 10-100 individual colonies. The left half of the plate shows a pure culture of *H. pylori* bacteria. (B) The presence of contaminating bacteria from the mouse gastric microbiota (left) or large numbers of *H. pylori* colonies (right) can complicate the enumeration of *H. pylori* CFUs. (C) Common examples of contaminating bacteria in gastric homogenate samples. Scale bar = 1.7 cm.

Figure 4: PCR detection of *H. felis* infection in gastric biopsies using oligonucleotides targeting the *ureB* gene. A specific oligonucleotide pair was designed to recognize and bind to homologous sequences in both *H. felis* and *H. pylori ureB* genes. These primers were validated using genomic DNA from *H. pylori* SS1 (lane 2) or *H. felis* (lane 3). Deionized water was included as a negative control (lane 1).

Figure 5: **Representative images of H&E-stained stomach sections from WT and KO mice at 6 months' post-infection with *H. felis*.** Paraffin-embedded tissue sections were stained with H&E. WT mice receiving BHI broth alone (control) had a normal gastric epithelium and no significant inflammation. In contrast, WT animals with chronic *H. felis* infection displayed moderate levels of inflammation and mucosal thickening which was further exacerbated in *H. felis*-infected KO animals. Tissue sections from *H. felis*-infected KO mice exhibited the presence of mucosal lymphoid follicles (*), cellular infiltrates (→), gland atrophy (▸) and hyperplasia. Scale bar = 100 µm.

Figure 6: Representative images of Giemsa-stained gastric sections from C57BL/6 WT mice at 3 months' post-infection with *H. felis*. Paraffin-embedded tissue sections were stained with Giemsa. Arrows indicate the presence of *H. felis* in the gastric glands. Scale bar = 200 µm.

DISCUSSION:

This protocol describes the use of an *in vivo* mouse model for *Helicobacter* infection. The critical steps of the procedure are the: 1) preparation of *Helicobacter* inocula containing viable and motile bacteria; 2) delivery of the appropriate numbers of bacteria to the mouse via intragastric gavage; 3) detection of bacterial infection by colony counting and/or PCR; and 4) processing of

gastric tissues to enable the assessment of histopathology in infected stomachs. Further suggestions for modifications, troubleshooting and technical considerations are discussed below.

The method of growing *Helicobacter* spp. using Blood Agar Base no. 2 supplemented with horse blood has been well established in our laboratory. However, alternate agar bases such as Brucella agar and Columbia blood agar can also be used[22]. It is important to ensure that only sterile glassware that is free of detergent is used to prepare the growth medium. Furthermore, to obtain optimal growth, *H. pylori* bacteria should be routinely subcultured on agar plates that have residual moisture and are not dry. When preparing *Helicobacter* spp*.* inocula for infection, it is vital to subculture *H. pylori* and *H. felis* strains every 1-1.5 or 2 days, respectively, to ensure bacterial viability. At every subculture, bacteria should be assessed for their viability and motility by phase contrast microscopy. A urease assay can also be routinely employed to discriminate between gastric *Helicobacter* spp. and other bacteria[23], however, it is important to realize that this assay detects both viable and non-viable *Helicobacter* bacteria. Following inoculation of animals, viable counts on *Helicobacter* suspensions must be performed to quantitate numbers of viable bacteria used for infection. As *H. felis* does not reproducibly form isolated colonies on agar medium[15], estimation of bacterial numbers is performed using phase contrast microscopy. Quantification of bacterial numbers by optical density measurement (A_{600}) alone is inaccurate as this method does not discriminate between viable and non-viable bacteria. This method should not be used in *Helicobacter* research without rigorous optimization, as described above (Section 1.5).

When performing *Helicobacter* infection studies, it is crucial to consider the optimal mouse and *Helicobacter* strain, as well as the length of infection, to suit the purpose of the experiment. It is also essential to regularly confirm that the animals used for experimentation are indeed *Helicobacter*-free using genus-specific PCR primers[24]. The presence of other enteric *Helicobacter* species, such as *Helicobacter bilis*, *Helicobacter hepaticus* or *Helicobacter muridarum,* may alter the disease susceptibility of mice and introduce confounding factors into *in vivo* studies[25,26]. It is also advisable to include a mock treatment control group of animals (*i.e.*, fed broth only) in initial screening experiments to investigate the effects of the normal microbiota on *Helicobacter* colonization and pathogenesis.

Post-euthanasia, *H. pylori* colonization in murine stomachs can be measured by viable counting. HBA plates used for colony counts should be supplemented with bacitracin and naladixic acid in addition to the modified Skirrow's selective supplement, to restrict the growth of bacterial species from the normal gastric microbiota and hence prevent contamination[27]. *H. felis* does not always form colonies, but instead tends to form swarming growth on agar plates[15,28]. Therefore, PCR and qPCR are normally employed to determine the presence and levels of *H. felis* colonisation, respectively[29,30]. In section 4.2, we introduced a simple and quick PCR method to confirm colonization by *H. felis* in the murine stomach using a pair of primers, which have been validated to target a 325-bp region of *H. felis* and *H. pylori ureB* genes. Using the PCR conditions described above, it is possible to discriminate between infection by these gastric *Helicobacter* spp. and urease-producing enterohepatic *Helicobacters.* Other genes that have

been validated for PCR detection of gastric *Helicobacter* infection include the 16s rRNA and flagellin B (*flaB*) genes[15,29,30].

Finally, we have described the use of two powerful staining techniques: H&E staining, to assess histopathological changes in the stomach post-infection; and Giemsa staining, to detect *H. felis* infection. To obtain optimal staining, it is essential to ensure that tissues have been preserved, processed and embedded in the correct orientation. Additionally, only freshly prepared solutions and filtered stains must be used during this process. Tissue sections can be stored indefinitely and utilized for more specific analysis of gastric pathology via immunofluorescence or immunohistochemistry. Some other common measures of gastric inflammation and disease include: immune cell recruitment (anti-CD45 staining); mucosal thickening/destruction (Periodic Acid Schiff/Alcian blue staining); epithelial cell proliferation (proliferating cell nuclear antigen, PCNA/Bromodeoxyuridine, BrDU staining); or cellular apoptosis (TUNEL staining). The lymphoid follicles observed in the H&E-stained tissues of *H. felis*-infected mice can be confirmed by immunohistochemistry, using antibodies directed against B ($B220^+$) and T cell ($CD3^+$) antigens[31].

In summary, animal models of bacterial disease provide valuable tools in the field of infection biology. The protocols of intragastric gavage and processing of stomach tissues provided here may be adapted to mouse infection models involving other enteric pathogens.

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DISCLOSURE:

The authors have nothing to disclose.

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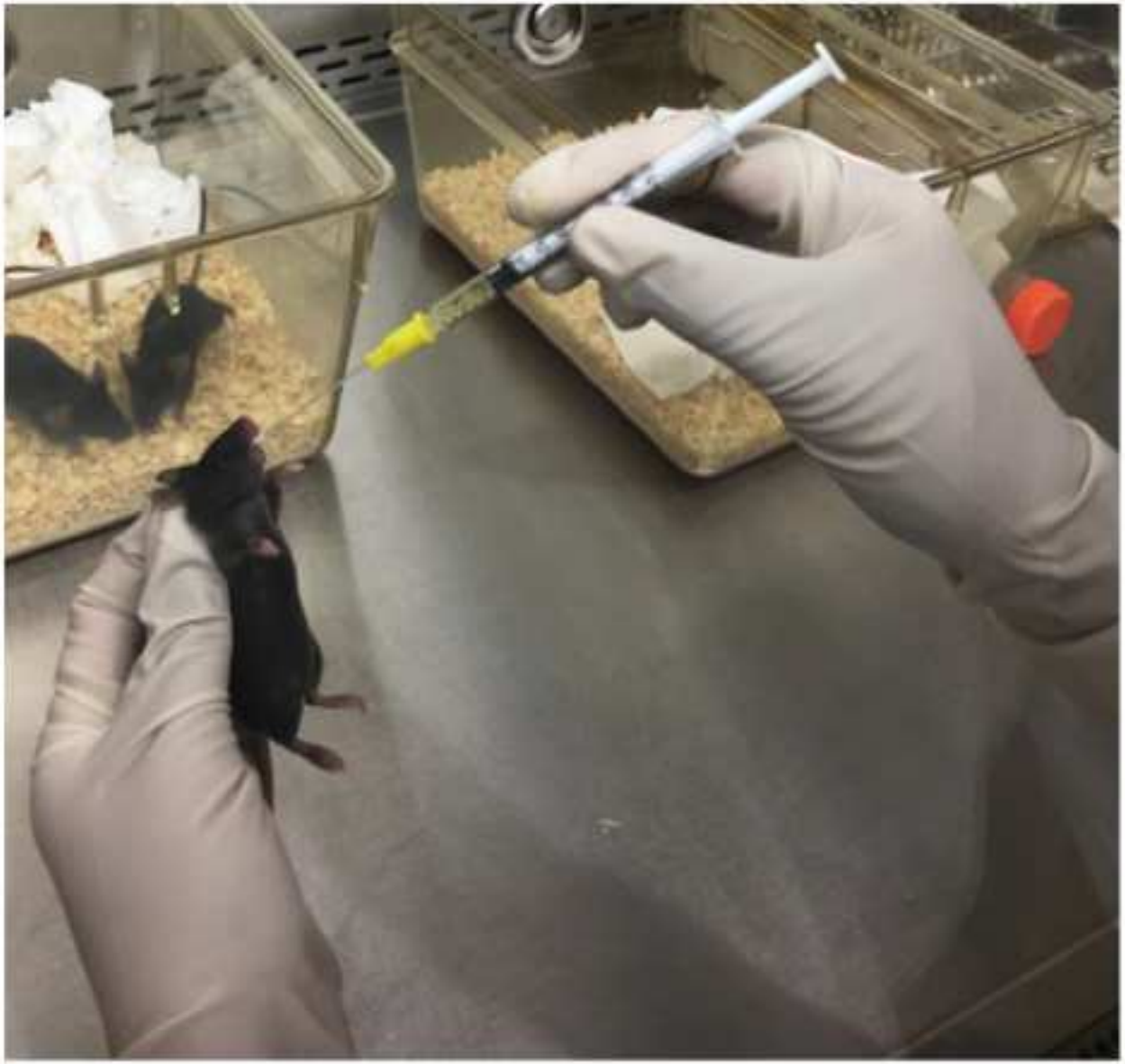

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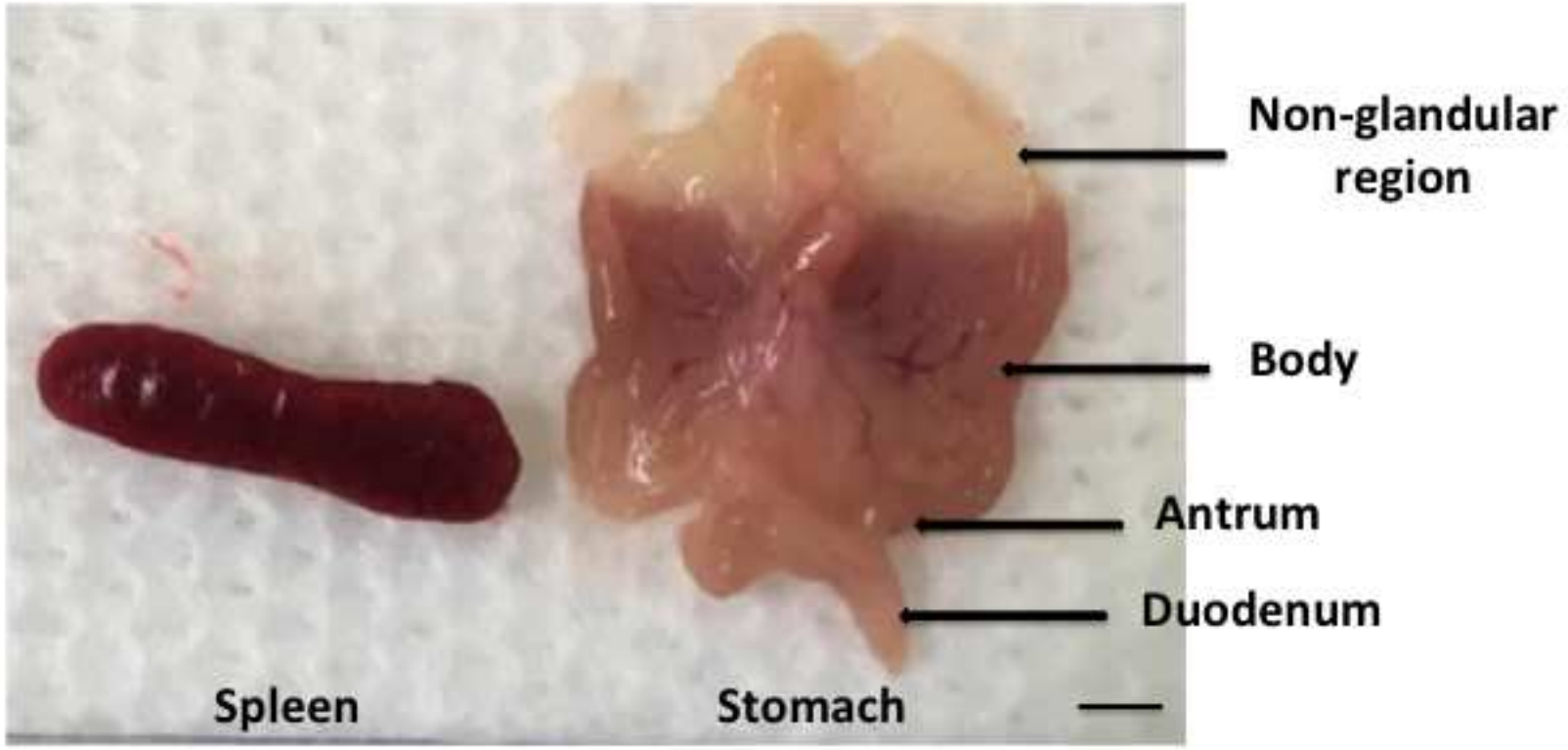

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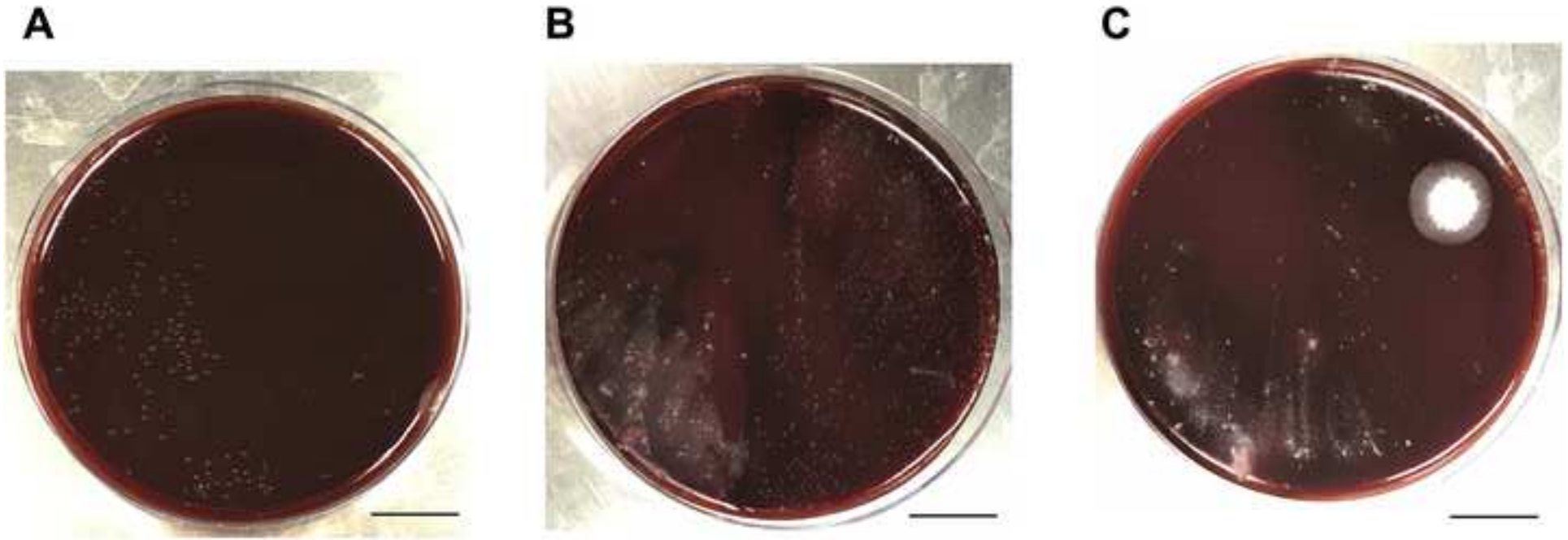

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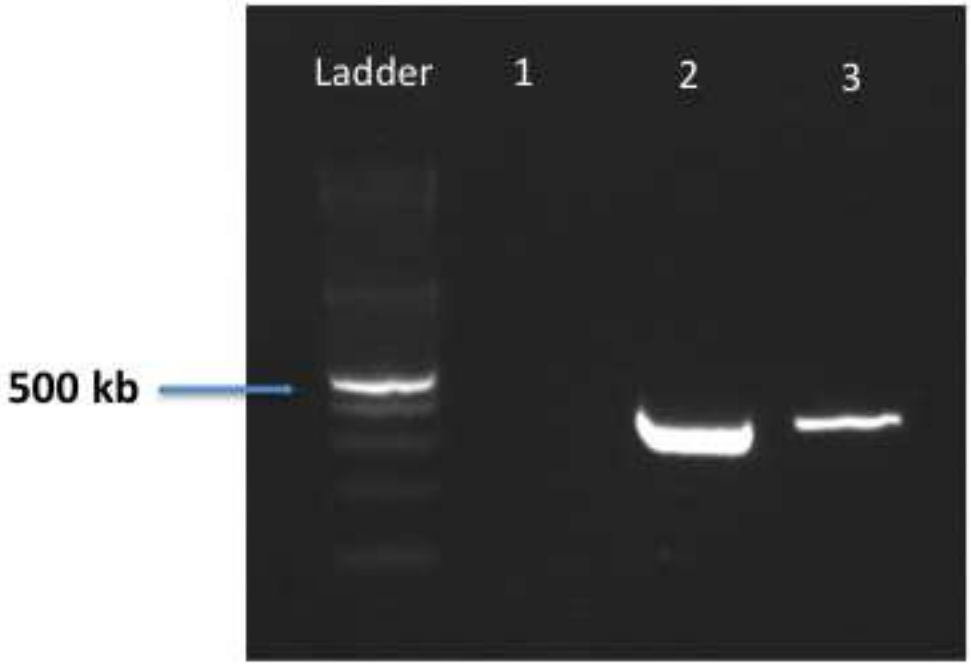

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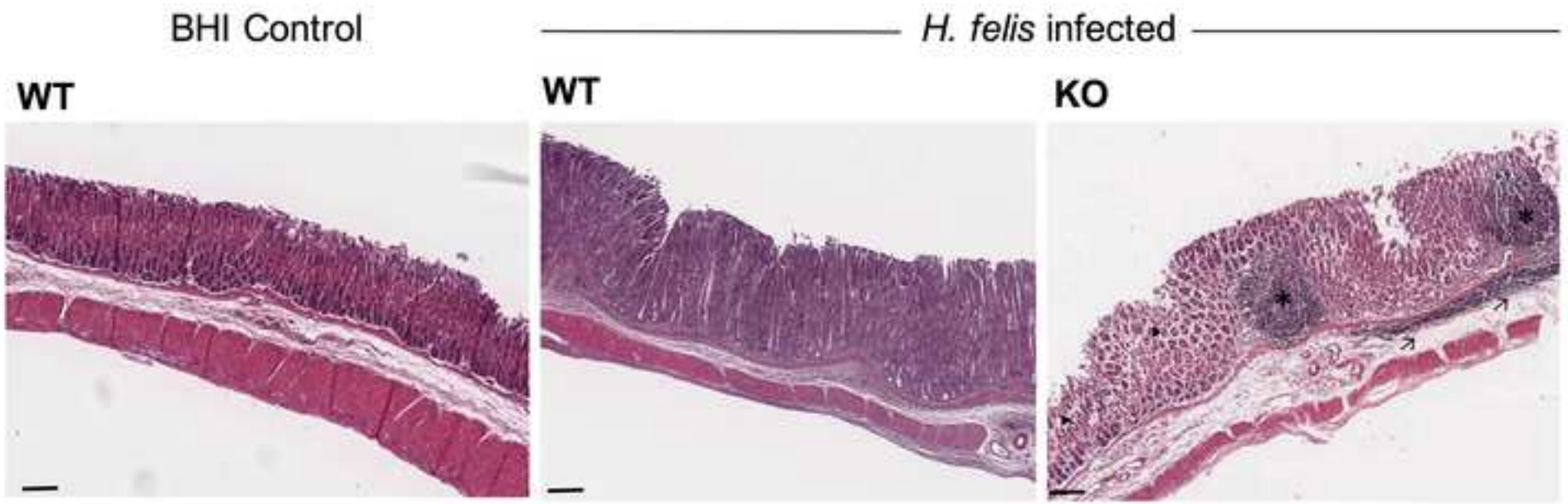

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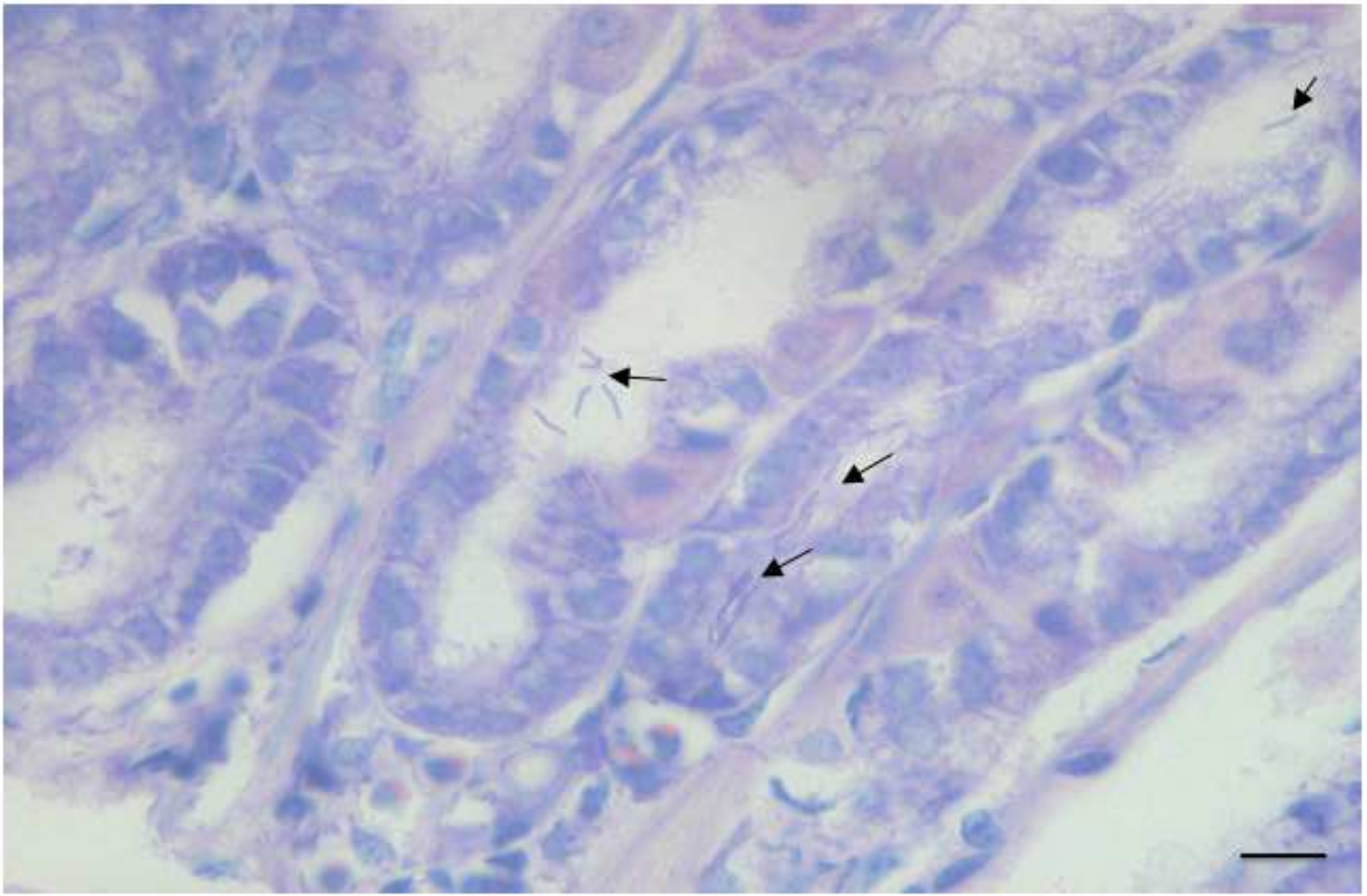

Materials Table

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Name of Material/ Equipment

Bacteriological reagents

Oxoid Blood Agar Base No.2
Premium Defibrinated Horse blood
Bacto Brain Heart Infusion Broth
CampyGen gas packs

Histological reagents

Formalin, neutral buffered, 10%
Absolute alcohol, 100% Denatured
Isopropanol (2-propanol)
Xylene (sulphur free)
Mayer's Haematoxylin
Eosin, Aqueous Stain
Wright-Giemsa Stain, modified
Histolene
DPX mounting medium

Molecular biology reagents

Qubit dsDNA BR Assay Kit
Oligonucleotides
GoTaq Flexi DNA Polymerase
dNTPs
Molecular Grade Agarose
Sodium Hydrogen Carbonate
Magnesium Sulphate Heptahydrate

Antibiotics

Vancomycin
Polymyxin B
Trimethoprim (≥98% HPLC)
Amphotericin
Bacitracin from *Bacillus licheniformis*
Naladixic acid

Other reagents

Methoxyflurane (Pentrhox)
Paraffin Wax

Equipment and plasticware

Oxoid Anaerobic Jars
COPAN Pasteur Pipettes
Eppendorf 5810R centrifuge
23g precision glide needle
Parafilm M
Portex fine bore polythene tubing
Plastic feeding catheters

1 ml tuberculin luer slip disposable syringes

Eppendorf micropestle for 1.2 - 2 mL tubes

GentleMACs Dissociator
M Tubes (orange cap)
Qubit Fluorometer
Sterile plastic loop

Cold Plate, Leica EG1160 Embedding System

Tissue-Tek Base Mould System, Base Mold 38 x 25 x 6
Tissue-Tek III Uni-Casette System
Microtome, Leica RM2235
Charged SuperFrost Plus glass slides

Company	Catalog Number
Thermo Fischer Scientific	CM0271B
Australian Ethical Biologicals	PDHB100
BD Bioscience	237500
Thermo Fischer Scientific	CN0035A/CN0025A
Sigma Aldrich	HT501128
ChemSupply	AL048-20L-P
Merck	100995
ChemSupply	XT003-20L
Amber Scientific	MH-1L
Amber Scientific	EOCA-1L
Sigma Aldrich	WG80-2.5L
Grale Scientific	11031/5
VWR	1.00579.0500
Thermo Fischer Scientific	Q32850
Sigma Aldrich	
Promega	M8291
Bioline	BIO-39028
Bioline	BIO-41025
Univar (Ajax Fine Chemicals)	A475-500G
Chem-Supply	MA048-500G
Sigma Aldrich	V2002-1G
Sigma Aldrich	P4932-5MU
Sigma Aldrich	T7883
Amresco (Astral Scientific)	E437-100MG
Sigma Aldrich	B0125
Sigma Aldrich	N8878

Medical Developments International	Not applicable
Paraplast Plus, Leica Biosystems	39601006
Thermo Fischer Scientific	HP0011/HP0031
Interpath Services	200CS01
BD Bioscience	301805
Bemis, VWR	PM996
Smiths Medical	800/100/200
Instech Laboratories	FTP20-30
BD Bioscience	302100
Sigma Aldrich	Z317314
Miltenyi Biotec	130-093-235
Miltenyi Biotec	30-093-236
Thermo Fischer Scientific	Q33216
LabServ	LBSLP7202
Leica Biosystems	Not applicable
Sakura, Alphen aan den Rijn	4124
Sakura, Alphen aan den Rijn	4170
Leica Biosystems	
Menzel Glaser, Thermo Fischer Scientific	4951PLUS4

\

Comments/Description

Dissolve in deinonized water prior to sterilization

Dissolve in deinonized water prior to sterilization

Filter before use
Filter before use
Dilute before use (20% Giemsa, 80% deionized water)

The annealing temperature of *ureB* primers used in this study is 61°C
Kit includes 10X PCR buffer and Magnesium Chloride
Dilute to 10mM in sterile nuclease free water before use

Dissolve in deionized water
Dissolve in deionized water
Dissolve in 100% (absolute) Ethanol
Dissolve in deionized water
Dissolve in deionized water
Dissolve in deionized water

Collect bacterial pellets by centrifugation at 2,200 rpm for 10 mins at 4°C

Autoclavable polypropylene pestles used for stomach homogenization

Use a pre-set gentleMACS Programs for mouse stomach tissue

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Re. Manuscript submission by D'Costa *et al.*

Melbourne, 16th April 2018

Dear Editor,

Please find below responses to the peer-reviewers' comments for our manuscript entitled: "Mouse models of *Helicobacter* infection and gastric pathologies."

Reviewer #1:

Manuscript Summary:

This manuscript and future video to be produced and associated with it describe the *in vitro* growth of gastric *Helicobacter* species, *H. pylori* and *H. felis*, and their use to infect mice as models for gastric *Helicobacter* infections. This is followed by methods to determine the magnitude of infection and histologic changes in the gastric mucosa of infected mice. Over the past 20 + years several publications have appeared detailing methods such as these including an entire monograph published in 1996 by W. B Saunders and edited by A. Lee and F. Megraud. (This monograph is out of print but still available at sites such as Amazon.com and in numerous biomedical libraries.) Surprisingly, many of the methods have not changed very much over the past 20 years notwithstanding the fact that the widely used Sydney strain of *H. pylori* was not introduced until 1997 and PCR techniques for quantitation, especially of *H. felis* were not readily available at that time. What makes the present manuscript relevant is the inclusion of videos which can be quite useful, especially for laboratories with no previous experience in mouse models of *Helicobacter* infections.

Minor Concerns:

1. In the discussion of this ms (line 443) the authors mention the rapid urease test which has been used both clinically in humans and experimentally in animal models to monitor gastric helicobacter infections. This reviewer does not dispute the authors comments that this test can detect both viable and non-viable organisms and should not be used to quantify the magnitude of a gastric *Helicobacter* infection of mice. However, this reviewer as well as many other authors have used the rapid urease test and tests for catalase and oxidase as simple screening assays to confirm that colonies or other outgrowth (such as observed with *H. felis*) from mouse stomach biopsies in fact represent gastric *Helicobacter* infections rather than some other bacterial constituent of the murine gastric microbiota. On some occasions, even

using selective culture plates and particularly when infecting gene targeted "knockout mice" from non-commercial vendors this reviewer has observed outgrowth of non-*H. pylori* bacterial colonies with a gross appearance very similar to authentic *H. pylori*, but exhibiting negative results for one or more of these rapid screening tests. Thus it would be useful and quite simple to include demonstration of these screening tests in the videos to be produced with this ms. In the absence of positive results on all or any of these screening tests, further confirmation of whether recovered bacteria are in fact *Helicobacter* spp could then be accomplished by PCR analysis as described in section 4.2 of this ms (lines 235-252).

We have now edited the manuscript as follows: "*H. pylori* colonies and *H. felis* growth on plates can be distinguished from those of other members of the murine gastric microbiota using the urease, catalase and oxidase tests. These gastric *Helicobacter* spp are positive for all three tests."

2. In section 1.5 Growth and Preparation of Bacterial Inocula (lines 134-137) and again in the discussion (lines 449-451) the authors advise against the use of optical density to estimate the number of *H. pylori* or *H. felis* to be used to inoculate mice but instead to view wet mounts and estimate that under 100X magnification "1 bacterium per field = approximately 10e6 colony forming units" etc (lines 122-127). Particularly at the lower end, such estimates can be very crude especially if a laboratory's microscopes do not readily achieve 100X total magnification (e.g. a 10X objective and a 12.5X eyepiece = 125X etc.) It would be better to define the number of viable bacteria counted within the grids of a standard hemacytometer for a much more precise estimate.

We apologise for the error; bacterial numbers are actually estimated using a 100 X objective, under oil immersion. As haemocytometers are not usually compatible with 100 X/oil immersion objectives, it is not possible to reach the level of magnification required to count individual *H. pylori* bacteria. Conversely, it is true that this method works well with *H. felis* (which is larger than *H. pylori*) and was standard practice in the PI's laboratory for many years but, in our hands, does not produce better results. Nevertheless, we agree that this is worth mentioning to the inexperienced researcher and so have amended the manuscript as follows: "In the case of *H. felis*, which is a much larger bacterium than *H. pylori*, it is possible to use a haemocytometer to accurately count the numbers of viable bacteria." and "When using a haemocytometer, calculate CFU/mL using the following formula: CFU/mL = (average number of bacteria in a 4 x 4 field) x (dilution factor) x (10^4)."

3. Alternatively, to prepare an inoculum to infect mice, after thawing bacteria and colonies appear on appropriate agar plates incubated under micro-anerobic conditions as described in the present ms, *H. pylori* can be passed into liquid BHI (as described on lines 113-114 of the ms) or Brucella broth with added fetal calf serum and then incubated in standard flat bottom/side tissue culture flasks in a CO_2 incubator at ca 10% CO_2. Using this method, it is possible to obtain a growth curve of optical density

versus viable counts of *H. pylori* by measuring the number of colony forming units in an aliquot of the liquid culture obtained every 4-6 hr vs optical density over 2-3 days (detailed in Garhart *et al*, *Infection and Immunity* 70: 3529-3538, 2002 and Blanchard and Nedrud, Laboratory Maintenance of *Helicobacter* Species, Current Protocols in Microbiology, Wiley, 8B.1.1-8B.1.19, 2012). Once a suitable growth curve is obtained, (which must be done for any laboratory using this method to account for inter-laboratory differences) it is a simple matter to measure the optical density of a sample from the flask and then to refer to the growth curve to estimate the number of viable bacteria in the inoculum. It is also easy to prepare new subcultures when needed by simple inoculation into new tissue culture flasks containing fresh broth/FCS. This method has the added advantage that the "health/viability" of the bacterial cultures can be rapidly monitored without removing from and requiring additional anaerobic jars and CampyGen sachets. Flasks are simply removed from the CO_2 incubator and examined using an inverted microscope at high magnification looking for high motility, spiral/helical morphology, lack of dead or coccoid bacteria, and lack of heavy clumps which might give a false estimate of bacterial numbers. *H. felis* does not readily grow under these conditions but can be grown in agitated liquid cultures inside of anerobic jars positioned on a laboratory shaker. As the authors of the present manuscript caution, however, once thawed from frozen stocks it is important to frequently subculture either plate grown or liquid culture grown *Helicobacter* spp to ensure that early-mid log phase cultures with primarily viable (and not coccoid or dead) bacteria are used for a mouse inoculum. It is also important to confirm the estimates of *Helicobacter* bacteria actually in the inoculum used to infect mice by quantitative culture of a sample of the inoculum (lines 130-132 of this ms).

From our experience, as well as anecdotally, not all *H. pylori* strains grow well in CO_2 incubators and hence why we did not include this method. Nevertheless, we agree that when properly optimized, this method has its merits. The manuscript has been modified as follows: "It is, however, possible to use optical density values as a means of estimating the numbers of viable *H. pylori* bacteria in inocula, but in this case, it is first necessary to generate a growth curve. For this, the A_{600} values of *H. pylori* cultures are monitored over time and correlated directly against the numbers of viable bacteria, determined by plate counting. A convenient method for performing such growth curve determinations is to culture bacteria in liquid medium (Section 1.2), using standard flat bottom tissue culture flasks placed in a 10% CO_2 incubator. The numbers of CFUs, determined from aliquots of the cultures obtained every 4-6 h over 2-3 days, are then compared to the corresponding A_{600} values (Blanchard and Nedrud, Laboratory Maintenance of *Helicobacter* Species, Current Protocols in Microbiology, Wiley, 8B.1.1-8B.1.19, 2012). Importantly, growth curves must be generated for each *H. pylori* strain, as these may grow at different rates and, furthermore, not all strains grow well in 10% CO2."

Also, we have added the following statement to the Discussion:

"Quantification of bacterial numbers by optical density measurement (A_{600}) alone is inaccurate as this method does not discriminate between viable and non-viable bacteria. This method should not be used in *Helicobacter* research without rigorous optimisation, as described above (Section 1.5)."

Reviewer #2:

Manuscript Summary:

This paper clearly describes the mouse model of *Helicobacter pylori*/*Helicobacter félis* infections. I would recommand this paper for scientifics who need to develop such model.

Minor Concerns:

Can the authors check the size of the *ureB* fragment amplified in the PCR, indeed I found a size of 325bp and not 342.

We thank the reviewer for detecting this error. This information has been corrected in the revised manuscript.

Can the author add to Figure 5 a picture of a non inflammed stomach mucosa.

This image has now been added to a revised version of Figure 5.

Can the author specify the KO gene in their KO mice, this is not fair for the reader to keep it confidential.

The mouse KO model has not yet been published and its identity is not, we believe, relevant to a methods article of this type.

Can the author describe/add gram staining in section 1.3 ? In my point of view it is critical to verify that the culture is only composed of Hp or Hf and the wet mount is not appropriate to check the purity of the bacterial suspension.

We have added the sentence: "Culture purity can be confirmed by performing a Gram-stain."

Can the author Figure 3C, it is not really positive for the paper to show contamination (very personnal view)

Although the medium used to grow *Helicobacter* spp. from mouse stomachs is highly selective, it is quite common to also isolate non-*Helicobacter* spp. on this medium. For this reason, we think that it is important to stress this point to inexperienced workers. Reviewer #1 made a similar comment about this in his/her comments.

We trust that we have satisfactorily addressed the reviewers' minor concerns and that the manuscript is now suitable for publication.

Yours Sincerely,

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