**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 (104).”**

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 CO2 incubator at ca 10% CO2. 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 CO2 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 CO2 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 A600 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% CO2 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 A600 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 (A600) 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,



Professor Richard L. FERRERO

NHMRC Senior Research Fellow

Hudson Institute of Medical Research

Monash University

27-31 Wright St., Clayton (3168)

Victoria

Australia