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**BIOMEDICAL SCIENCES**

# PCR-based detection of *Helicobacter* spp. in animal facilities of a University in Rio de Janeiro, Brazil

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**Abstract:** Pathogenic microbial detection and control in laboratory animal facilities is essential to guarantee animal welfare, data validity and reproducibility. *Helicobacter* spp. are known to affect mice health, what may interfere with experimental outcomes. This study aimed to screen for *Helicobacter* spp. in mice from animal facilities in Rio de Janeiro, Brazil using a PCR-based method. Primers designed to specifically identify *Helicobacter* spp. were used to amplify feces or intestine DNA extracted of mice from four different animal facilities. The expected 375 base pairs (bp) amplicon was purified, sequenced and a similarity of 95% was observed when compared to deposited sequences of *H. hepaticus* and *H. bilis*. In our screening, *Helicobacter* spp. was detected in ~59% of fecal and ~70% of intestine samples. Our study is the first to screen for *Helicobacter* spp. in mouse facilities of a Rio de Janeiro University using a low cost, rapid molecular diagnostic test. Although *Helicobacter* spp. screening is not mandatory according to Brazilian animal welfare regulation it is recommended by institutional animal health monitoring programs guidelines worldwide, including ARRIVE, AAALAC and FELASA.

**Key words:** Diagnostic, screening, laboratory mice, *Helicobacter*.

## INTRODUCTION

The use of rodents as experimental models in basic and pre-clinical research has been essential for scientific progress. Reproducible research requires laboratory animals free of diseases and other conditions that could interfere with experimental outcome. Infections that naturally occur in mice, even when subclinical, may influence animal's physiology, immunity and behavior. Therefore, even in the absence of clinical signs, experimental rodents may become inadequate for research due to microorganism infections that may be a threat mainly to immunosuppressed mice (Kullberg et al. 1998, Whary & Fox 2006, Besselsen et al. 2008, Sharp et al. 2008, Chichlowski & Hale 2009, Pritchett-Corning et al. 2009, Mahler Convenor et al. 2014). Although Helicobacter infection in C57/BL6 mice are usually asymptomatic, other strains such as A/JCr, BALB/c, SJL, B6C3F1, C3H/He, Rag-deficient and SCID may developed inflammatory bowel disease (IBD), intestinal cancer, hepatitis, and hepatocellular carcinoma (Whary & Fox 2006, Fox et al. 2011). Therefore, the establishment of animal health status monitoring routines is indispensable (Mahler Convenor et al. 2014, Bracken et al. 2017).

*Helicobacter* bacteria, a genus that includes about 45 species, are among the microorganisms that infect laboratory rodents (Mahler Convenor et al. 2014, Pere-Vedrenne et al. 2017, Menard & Smet 2019). While in humans, *Helicobacter pylori* infection is associated with gastric diseases, in

rodents *Helicobacter* spp. may infect the intestine (cecum and colon), stomach, gallbladder and liver; causing diseases in specific mouse strains (Chichlowski & Hale 2009, Pere-Vedrenne et al. 2017, de Brito et al. 2019). The disease-causing species in susceptible mice include *H. rodentium, H. bilis. H. ganmani, H. muridarum and H. typhlonius* (Duangchanchot et al. 2014, Pere-Vedrenne et al. 2017, Menard & Smet 2019). In mice, *Helicobacter* spp. infection is associated with gastrointestinal and inflammatory bowel disease as well as liver, gastric and colon cancers. In immunodeficient animals, other gastrointestinal manifestations caused by *Helicobacter* spp. infection include thyphlocolitis, hepatitis and gastritis. *Helicobacter* infection may also affect mice reproduction (Sharp et al. 2008, Chichlowski & Hale 2009, Yang et al. 2013). For example, infection with *H. typholonius* reduced the number of embryos per female (Bracken et al. 2017). In addition, co-infection with *Helicobacter* may also facilitate several diseases. For instance, lung inflammation and injury as well as the growth of *Mycobacterium tuberculosis* are increased in mice naturally infected with *H. hepaticus* (Majlessi et al. 2017). Importantly, *Helicobacter* bacteria are shed in feces leading to horizontal transmission through fecal-oral contact (Whary & Fox 2006).

*Helicobacter* spp. are highly prevalent bacteria in animal facilities worldwide (Taylor et al. 2007, Wasimuddin et al. 2012) and were previously detected in wild rodents in Brazil (Comunian et al. 2006). To our knowledge, the prevalence of *Helicobacter* infection in laboratory rodents in Brazil has never been studied. The present study aimed to detect *Helicobacter* spp. bacteria in rodents of animal facilities of the Universidade Federal do Rio de Janeiro (UFRJ), Brazil using a PCR-based diagnostic test.

## MATERIALS AND METHODS

BALB/c or C57BL/6 mice between 15 and 22 weeks of age (n=57; mean age=19 weeks) were randomly selected from four different UFRJ animal facilities (AF-A – AF-D) (Table I). According to Animal Ethics Committee of UFRJ protocol 092/15 mice were euthanized by $CO_2$ prior to colon collection. Recovered feces (1 fecal pellet/animal) and intestine sections were frozen in liquid nitrogen and maintained at -80$^o$C. For DNA extraction of fecal samples (about 1 cm pellet, ~50-80 mg) (Beckwith et al. 1997) or lower bowel (about 1cm of colon) samples were incubated in 100 µL of 25 mM NaOH (Isofar, 1326) for 1h at 98°C. Then, 400 µL of 10 mM pH 7.4 Tris buffer (Sigma, T1503) was added, debris were centrifuged and the samples were stored at -20$^o$C. Since feces may contain inhibitory substances that may lead to false negatives (Monteiro et al. 1997), we included DNA extracted from the feces of a mouse naturally infected with *Helicobacter* spp. (provided by Dr. Rovilson Gilioli, CEMIB, Unicamp) as positive control for the PCR reaction.

To investigate the presence of *Helicobacter* spp., we performed PCR assays using primers previously designed to recognize a conserved region of the 16S ribosomal RNA gene (16S rRNA), for the *Helicobacter* species (*H. hepaticus, H. bilis, H. muridarum and H. rappini*) (Beckwith et al. 1997). Primer-BLAST tool was used to compare the sequence of annealing primers to three different genomes: (i) *H. hepaticus* (NC_004917.1), (ii) *Mus musculus* (GCF_000001635.20), to search to nonspecific amplifiable targets and (iii) to *Escherichia coli* (GCF_000008865.2). The reaction mixture of PCR assay contained 0.5 µM of each primer (H276f: 5'-CTATGACGGGTATCCGGC-3' and H676r: 5´-ATTCCACCTACCTCTCCCA-3'), 10 mM dNTP's (Fermentas, R0199), 5x GoTaq® Buffer (Promega M891A), 1.5 mM $MgCl_2$ (Promega, A351H), and 0.025 U of Hotstart Taq polymerase

**Table I. Percentage of *Helicobacter* spp. infected mice as determined by *Helicobacter* genus-specific PCR analysis.**

| Animal facility | Mouse strain | N°. of positive samples / total tested (% of positive PCR) | |
|---|---|---|---|
| | | **Feces** | **Intestine** |
| **A** | C57BL/6 | 13 / 22 (59%) | 15 / 25 (60%) |
| **B** | C57BL/6 | 3 / 5 (60%) | 9 / 10 (90%) |
| **C** | BALB/c | 11 / 13 (84%) | 11 / 13 (84.6%) |
| **D** | C57BL/6 | 1 / 7 (14%) | 5 / 9 (55.6%) |
| **Total** | | **28 / 47 (59.6%)** | **40/57 (70.1%)** |

(Promega) and DNA. The amplification conditions used were: 94°C for 5' followed by 35 cycles of 2' at 94°C, 2' at 53°C and 30" at 72°C for 5' at 72°C. The PCR product was analyzed in 1% agarose gel (Sigma, A9539).

To determine whether the obtained PCR 375bp amplicon would correlate to the *Helicobacter* genome, first, samples of the amplified DNA were purified using a commercial kit (GE kit, 28903470). DNA sequencing was performed in the genomics facility of IBCCF, UFRJ. Sequencing reaction: 50 ng of DNA + 3.2 pmol of primer. DNA (H276f: 5'-CTATGACGGGTATCCGGC-3' was sequenced using an ABI 3130xl equipment and sequence comparison was performed using BLAST. Obtained sequence was compared to the 16S rRNA gene sequences of *H. hepaticus* (NC_004917.1), *H. bilis* (NZ_JMKW00000000.1), *H. muridarum* (NZ_JRPD00000000.2), *Mus musculus* (GCF_000001635.20) and *E. coli* (GCF_000008865.2) genomes using BLAST.

## RESULTS AND DISCUSSION

First, we performed a PCR reaction using a DNA previously isolated from a mouse infected with *Helicobacter* ssp. as a positive control. Different amounts of DNA (0.01, 1, 10 and 100ng) were analyzed and a single amplicon of 375 bp was detected in the three higher concentrations of DNA tested (Figure 1). The successful amplification using 10 ng and a detectable weak band using 1 ng of DNA indicated a sensitivity that allows amplification of DNA extracted from feces or tissue. Analysis of the sequence showed a ~95% similarity between this amplicon and the sequence of the 16S rRNA gene of *H. hepaticus*, *H. bilis* and *H. muridarum* deposited in NCBI database, further validating the specificity of the PCR assay. To evaluate primers specificity to *Helicobacter* species, we extracted DNA from *E. coli* bacteria using the same NaOH protocol described above. Following PCR, no amplification was observed with the input of 1, 10 or 100 ng of *E. coli* DNA (Figure 2).

To analyze the presence of *Helicobacter spp.* in different animal facilities, we collected feces or intestine DNA samples from 57 randomly selected mice and performed PCR analysis (Figure 3). Due to sampling limitations, not all animals were tested for both feces and intestines. We found that 59.6% (28 of 47) of

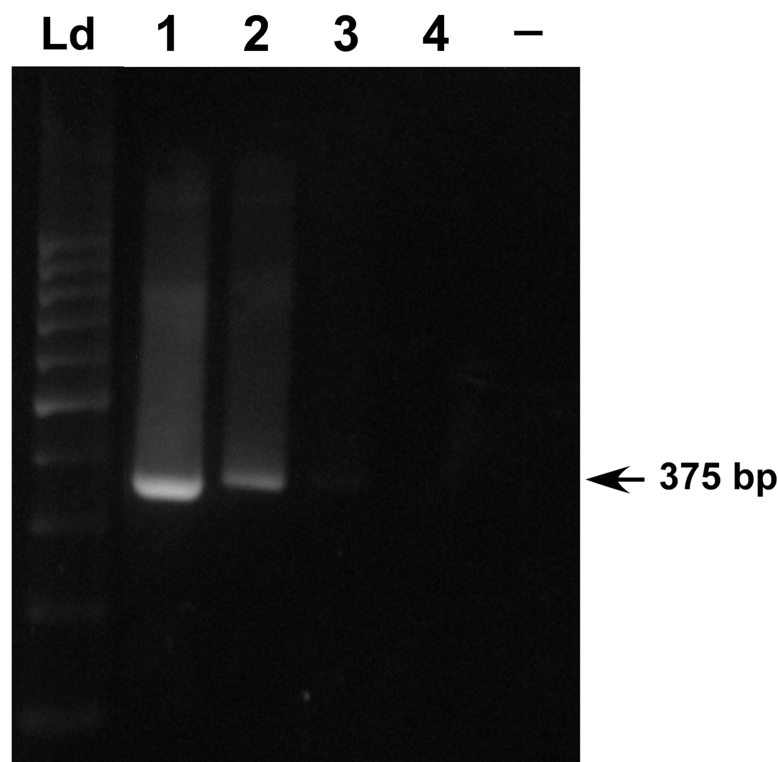


**Figure 1.** Sensitivity of the PCR-based assay for *Helicobacter* spp. detection: DNA was extracted from feces of a mouse that was previously diagnosed with *Helicobacter* infection was used as the input of the PCR. Labels: MW (molecular weight) = 100 bp DNA ladder; 1 = 100 ng; 2 = 10 ng; 3 = 1 ng and 4 = 0.1 ng and (-) = negative control; 1% agarose gel (ethidium bromide).

the feces and 70.17% (40 of 57) of the intestine samples contained detectable *Helicobacter spp.* DNA (Table I). According to the PCR-based diagnostic tests of the feces, the occurrence of *Helicobacter spp*. infection in the different animal facilities (AF) ranged from 14% (AF-D) to 84% (AF-C). The analysis of intestine samples revealed a proportion of *Helicobacter*-infected mice ranging from 55% (AF-D) to 90% (AF-B) (Table I). Although, the AF-D showed the lowest prevalence it still high index of contamination what may reflect a lack of an equivalent sanitary barrier as compared to other AF studied here (Mahler Convenor et al. 2014, Schlapp et al. 2018).

The use of living animal models in research requires periodic screening, therefore, laboratory mice should be monitored for parasite, bacteria, fungi and virus, using different methods such as culture, microscopy, PCR and serology (Moerth et al. 2008, Mahler Convenor et al. 2014). PCR is one of the most reliable methods for *Helicobacter* spp. detection (Chichlowski & Hale 2009, Casagrande Proietti et al. 2010), since culturing these microorganisms is a laborious procedure (Shames et al. 1995) and serological tests for *Helicobacter* spp. present low specificity (Whary & Fox 2006, Chichlowski & Hale 2009). Because the prevalence of *Helicobacter* spp. in rodents used for research throughout Brazil remains largely unknown, the validation and application of non-expensive tests to routinely screen mice colonies kept in Brazilian research facilities are particularly useful.

Here, we used primers against 16S rRNA able to identify several *Helicobacter* species that may infect mice. PCR sensitivity was determined using serial dilutions of NaOH-extracted DNA from feces and/or intestine. Efficient amplification demonstrated that less expensive methods of DNA extraction are useful for PCR-based screening tests of intestine and feces. In addition, screenings using a single

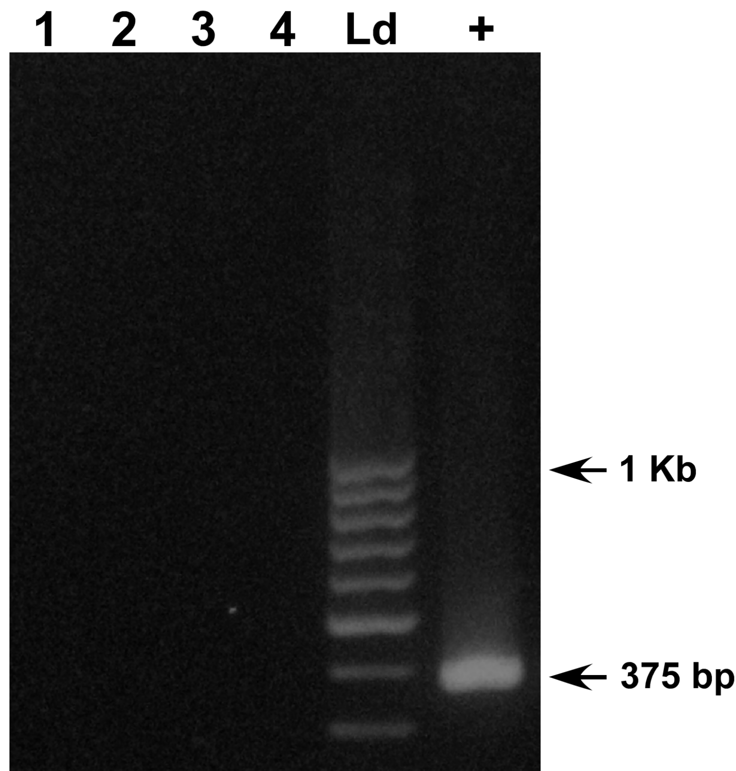


**Figure 2.** **Specificity of the PCR-based assay for *Helicobacter* spp. detection: DNA extracted from *E. coli* bacteria (1-4) and DNA extracted from feces of a mouse that was previously diagnosed with *Helicobacter* infection (+) were used as the input of the PCR. Labels: 1-4: respectively, 100, 10, 1 or 0.1 ng of *E. coli* DNA, MW = 100 bp DNA ladder; (+ positive control) 100 ng of *Helicobacter* spp. DNA; 1% agarose gel (ethidium bromide).**

pair of primers to detect several species of the same genus is advantageous because it minimizes time and costs (Battles et al. 1995, Shames et al. 1995). However, it should be noted that besides the four species investigated here, other *Helicobacter* species may infect rodents (Fox et al. 2011, Wasimuddin et al. 2012, Pere-Vedrenne et al. 2017, Menard & Smet 2019), thus it is important to adequate the choice of primers and PCR strategy.

A high prevalence of *Helicobacter* spp.-infected mice was observed in the facilities screened in this study. Overall, the percentage of positive samples was higher than 50%, therefore it is possible that a widespread contamination happened in the animal facilities studied. The successful use of feces is convenient, because it avoids euthanasia, however a study that performed side-by-side comparisons reported that in some cases feces is negative for *Helicobacter* while intestines are positive (Cao et al. 2020). More specifically, we observed that AF-D presented the lowest level of Helicobacter DNA positivity in the intestine (55.6%) and in the feces samples (14%). The difference between intestine and feces positivity may be because *Helicobacter* DNA detection may vary during infection and/or colonization. In a controlled experimental infection with *H. hepaticus*, bacterial DNA was detected in the feces of 100% of the animals at day 14 post-infection, while in the intestine and liver, bacterial DNA was detected only after 60 and 180 days, respectively (Cao et al. 2020). Thus, since the animals used in this study were chosen randomly it is impossible to determine the period of *Helicobacter* infection, and it is reasonable to assume differences in DNA availability in different periods of infection. Therefore, the use of both materials may be adequate. In addition, it is important to highlight that given the small differences in positivity within the facilities analyzed and the fact that

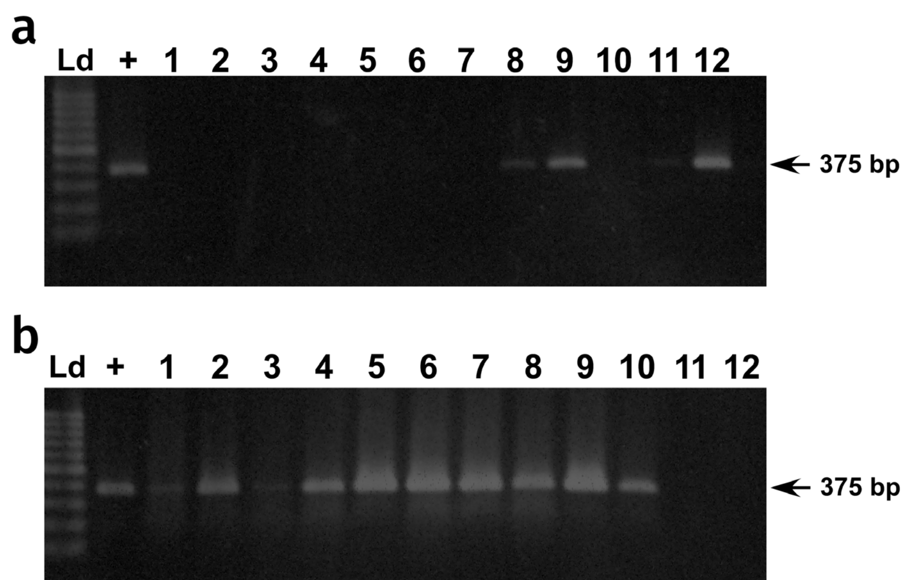


**Figure 3. PCR-based detection of *Helicobacter* spp. in feces and/or intestine of mice: Randomly selected representative samples of DNA extracted from mice feces (a) or intestines (b). (a) Labels: (+ positive control) *Helicobacter* spp. DNA; 1-12 = DNA extracted from mouse feces; (b) Labels: (+ positive control) *Helicobacter* spp. DNA; 1-12 = DNA extracted from mice intestine; 1% agarose gel (ethidium bromide).**

only two mice strains were screened, our data does not allow conclusions about associations between infection rates and mouse genetic background.

Regarding the relevance of our findings to basic research using mice as a model, it is now clear that different *Helicobacter* spp. may cause inflammatory bowel disease (IDB) in mice (Fox et al. 2011). Infection with *H. hepaticus* also led to colitis, which was associated with the increase of pro-inflammatory cytokines (Kullberg et al. 1998, 2006). Interestingly, infection with *H. pylori* may lead to the opposite consequences in IBD. Several studies have shown that *H. pylori* infection in mice may protect against IBD due to inflammatory down-regulation and immunological tolerance (Yu et al. 2018). Thus, it is crucial to determine the status of *Helicobacter* infection for studies modeling IBD in mice.

Notably, international councils, such as FELASA, ARRIVE, AALAS and AALAC, recommend the use of sentinel's animals for the health screening routine of rodent colonies (Lipman & Homberger 2003, Kilkenny et al. 2010, Newcomer 2012, Mahler Convenor et al. 2014). While the Brazilian National Council of Animal Experimentation Control (CONCEA 2016) highlights the importance of a health monitoring programs, it does not recommend the use of sentinel's animals. Taken into consideration that few companies provide services of microbiological screening tests for laboratory rodents in Brazil, our findings highlight the need for the development of accessible monitoring routines in order to prevent the use of infected animals.

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## Author contributions

GEMR and RAPM conceived and designed the experiments; GEMR, CCM and FJMS performed the experiments; GEMR, CCM, LOM and RAPM analyzed the data; GEMR, MF, LOM and RAPM wrote the manuscript.