

Chronic *Helicobacter pylori* Infection Does Not Significantly Alter the Microbiota of the Murine Stomach^{∇†}

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We examined the impact of *Helicobacter pylori* infection on the murine gastric microbiota by culture and terminal-restriction fragment length polymorphism and found that neither acute nor chronic *H. pylori* infection substantially affected the gastric microbial composition. Interestingly, the total *H. pylori* burden detected by real-time PCR was significantly higher than that revealed by viable counts, suggesting that the antigenic load sustaining *H. pylori*-induced gastritis could be considerably higher than previously believed.

Helicobacter pylori colonizes the stomachs of an estimated 50% of the world's population and is classified as a group 1 carcinogen (4, 15). Various animal models are currently used to study the pathogenesis of *H. pylori* infection, but there is little information on the gastric bacterial composition of rodents after *H. pylori* infection (1, 2, 7, 12). We therefore compared the gastric flora compositions, over time, of uninfected mice and of mice infected with *H. pylori* using conventional culture methods and terminal-restriction fragment length polymorphism (T-RFLP) analysis.

Six- to 8-week-old female C57BL/6 mice were reared under specific-pathogen-free conditions at the animal facility of the Department of Microbiology and Immunology, University of Melbourne. All animal experiments were approved by the University of Melbourne Animal Ethics and Experimental Committee and complied with relevant legislation. Statistical significance was assessed by the nonparametric Mann-Whitney U test, and two-tailed *P* values of less than 0.05 were considered statistically significant.

Preliminary results from culture of stomach homogenates and generation of 16S rRNA gene clone libraries from naïve mice confirmed that the murine stomach was highly colonized by lactobacilli, with the dominant species being *Lactobacillus reuteri* and *Lactobacillus murinus* (supplementary data), which is consistent with other studies (1, 2, 10, 12). In addition, T-RFLP data from an initial short-term *H. pylori* infection experiment with the SS1 strain (6) indicated that the gastric microbiotas of *H. pylori*-infected mice were similar to those of age-matched naïve mice, implying that an acute *H. pylori* in-

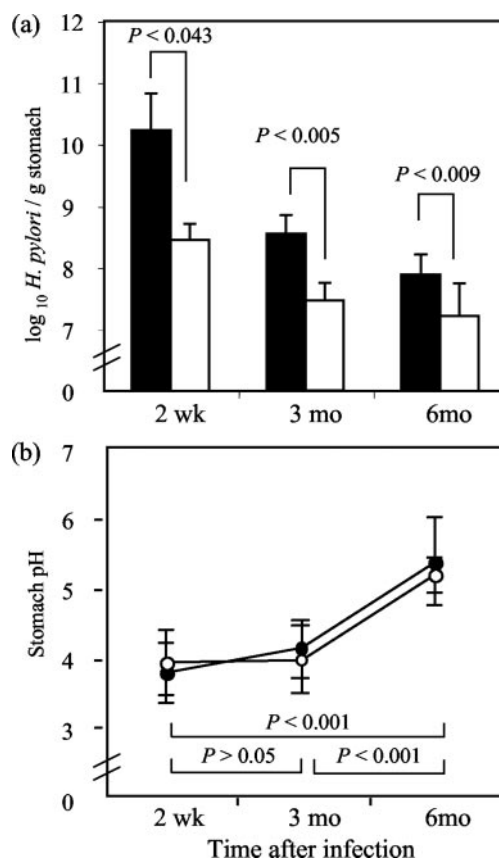


FIG. 1. *H. pylori* colonization and stomach pH over time. (a) *H. pylori* colonization was detected by real-time QPCR (filled bars) and culture (open bars) at 2 weeks, 3 months, and 6 months after infection. The means \pm standard deviations from groups of 9 to 10 mice are presented. (b) Stomach pHs of infected and naïve mice. Shown here are the mean values \pm standard deviations for 9 to 10 infected (●) and naïve (○) mice.

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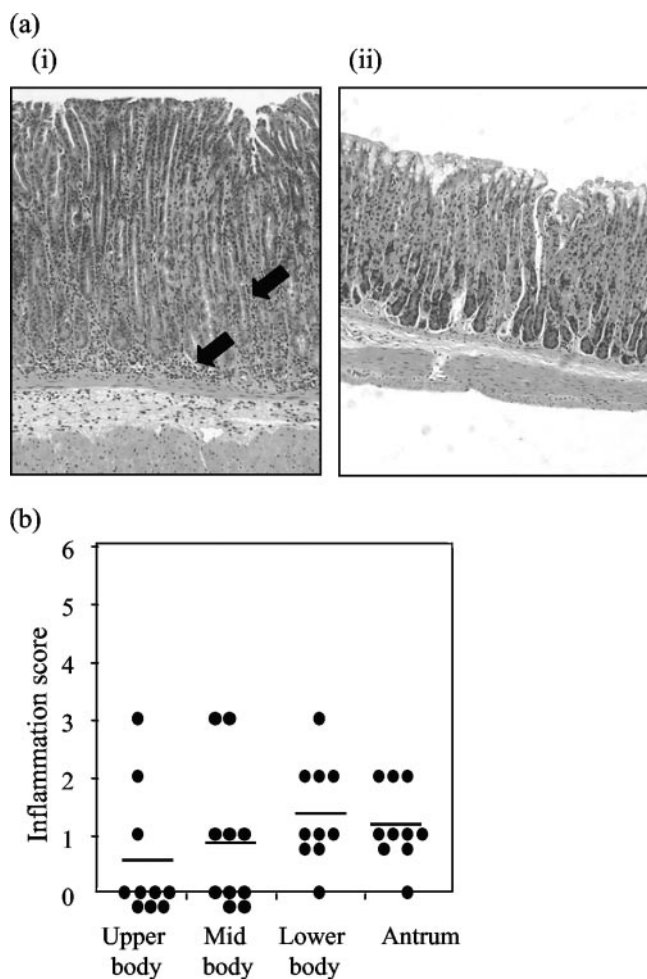


FIG. 2. Histological examination of inflammation in the stomach. Stomach sections from mice infected for 6 months with *H. pylori* were stained with hematoxylin and eosin and analyzed for pathology (13). (a) Representative hematoxylin- and eosin-stained sections from infected (i) and uninfected (ii) mouse stomachs. Arrows indicate lymphocyte infiltration. (b) Summary of inflammation scores from various stomach regions of *H. pylori*-infected mice. Stomachs from uninfected mice revealed no inflammation. The horizontal bars indicate the mean scores.

fection does not significantly affect the gastric microbiota (supplementary data). Based on these results, we sought to study the effects of progressive *H. pylori* infection by infecting mice with *H. pylori* and examining their microbiotas at 2 weeks and 3 and 6 months after infection (9 or 10 mice per group) by T-RFLP analysis and culture. In vitro growth conditions of *H. pylori* were as previously described (5); mice were also infected and *H. pylori* organisms from mouse stomachs were quantified by culture as previously described (5). Gastric *H. pylori* loads were also assessed by *H. pylori*-specific real-time quantitative PCR (QPCR), where *H. pylori*-specific primers (Hp547f, 5'-C TTAACCATAGAAGCTGCATTGAAACTAC-3'; Hp665r, 5'-GGTCGCCTTCGCAATGAGTA-3' [Geneworks Pty. Ltd., SA, Australia]) and 6-carboxyfluorescein (FAM)-labeled TaqMan MGB probe (Hp597p, 5'-TACCTCTCCCACACTCT-3' [Applied Biosystems, CA]) were designed using the Primer Express software (Applied Biosystems) based on the published

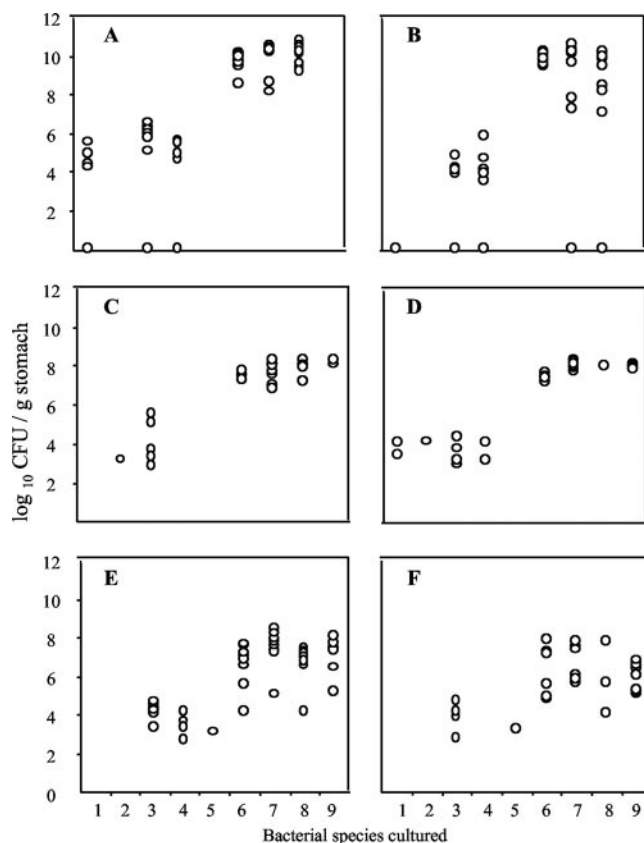


FIG. 3. Microbiotas cultured from the stomachs of mice with (A, C, and E) and without (B, D, and F) persistent *H. pylori* infection at 2 weeks (A and B), 3 months (C and D), and 6 months (E and F). Bacterial species identified by culture were as follows: column 1, *Escherichia coli*; column 2, *Moraxella osloensis*; column 3, *Actinobacillus muris*; column 4, *Pasteurella* sp.; column 5, *Enterococcus faecalis*; column 6, *Lactobacillus murinus*; column 7, *Lactobacillus reuteri*; column 8, *Lactobacillus gasseri*; and column 10, *Lactobacillus intestinalis*.

H. pylori SS1 16S rRNA gene sequence (GenBank accession no. AY456638), using QPCR conditions described elsewhere (9). As the *H. pylori* genome contains two copies of the 16S rRNA gene, the number of 16S rRNA genes detected by QPCR was halved to estimate the number of *H. pylori* genomes. All infected mice were colonized with *H. pylori* throughout the experiment, with levels of viable bacteria being comparable with those in other studies using the same mouse infection model (3, 6, 14), but the levels of *H. pylori* detected by QPCR was 5- to 60-fold higher than those detected by culture (Fig. 1a). The pH of surface gastric mucosal tissue was measured by gently pressing pH strips (Sigma, St. Louis, MO) on intact exposed mucosa and was performed to determine whether any observed change in gastric flora upon *H. pylori* infection was due partly to alterations in the stomach acidity, especially in chronic infections. Although gastric pH increased significantly from 2 weeks to 6 months in both infected and naïve mice, the pHs were not statistically different between the groups at any time (Fig. 1b). Mice infected for 6 months were also assessed for stomach inflammation by histology as described previously (5, 13), because infiltrating immune cells and

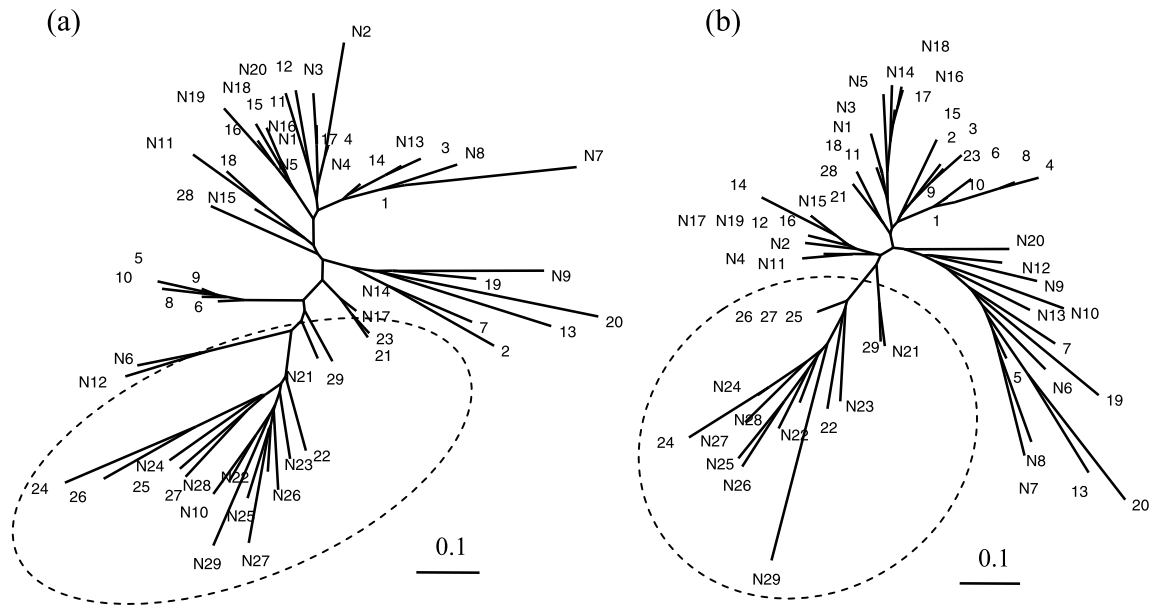


FIG. 4. Dendrograms generated from T-RFLP analyses using (a) a DraI/HhaI double digest or (b) a HaeIII digest of FAM-labeled PCR products. Scale bars indicate a 10% difference between individual samples. Numbers: 1 to 10, mice infected for 2 weeks; 11 to 19, mice infected for 3 months; 20 to 29, mice infected for 6 months; N1 to N10, naïve mice in the 2-week group; N11 to N20, naïve mice in the 3-month group; N21 to N29, naïve mice in the 6-month group. Dashed ovals indicate the apparent clustering of mice from the 6-month group.

damage to the gastric mucosal epithelium could affect the numbers and types of bacteria able to proliferate in the stomach with the existing *H. pylori* infection. Our results indicated that all infected mice had developed a lymphocytic infiltrate and showed inflammation in at least one region of the stomach examined but that none of the naïve mice developed gastritis (Fig. 2).

We then examined the gastric microbiotas of these mice by T-RFLP analysis and culture, where serially diluted stomach homogenates were plated on blood agar and incubated microaerophilically for 48 h. Culture results again confirmed the predominance of lactobacilli and indicated that the compositions of culturable bacteria in mice infected with *H. pylori* and in naïve mice appeared to be similar at all time points (Fig. 3). T-RFLP analyses were performed as described previously (11), with slight modifications. Two separate T-RFLP analyses were performed to reduce the likelihood of different bacterial species yielding the same T-RF size. A double digest using DraI and HhaI (Promega, WI) allowed us to discriminate between the *Lactobacillus* species detected in our preliminary studies, and the second analysis used HaeIII (Promega). Dendrograms generated from the resulting matrices using PHYLIP (Phylogeny Inference Package; University of Washington) and TreeView PPC (University of Glasgow, United Kingdom) software packages revealed an apparent cluster of animals in the 6-month group that were age but not infection status related (Fig. 4). In comparison, infected and naïve mice from the earlier time points (2 weeks and 3 months) did not exhibit any obvious clustering.

Our results suggested that neither acute nor chronic *H. pylori* infection appeared to alter the murine gastric microbial composition, contrary to findings of previous studies (1, 8).

Neither culture nor T-RFLP analyses indicated any distinct differences between infected and uninfected mice. Rather, statistical analysis of the mean Sorensen's distances and diversity indices (Shannon's and Simpson's) using T-RFLP data showed that the mice infected for 6 months were similar to the age-matched naïve mice (data not shown). Hence, the bacterial community composition was not dramatically affected by the persistent colonization of *H. pylori* in the stomach and *H. pylori*-induced inflammation in the gastric mucosa seen in chronically infected mice or by the apparent age-dependent decrease in stomach acidity. It should be noted, however, that more-extensive *H. pylori*-induced damage to the mucosa (e.g., atrophy and metaplasia) might have occurred had the infection progressed further (6). Lastly, lymphocytes (predominantly T cells) infiltrating the gastric mucosa during chronic *H. pylori* infection are presumed to be specific for viable *H. pylori*. However, the QPCR data reported here showed that a high proportion of the *H. pylori* organisms present in mouse stomachs are in fact nonculturable, suggesting an underestimation of available bacterial antigens contributing to *H. pylori*-induced gastritis.

In summary, we have shown that neither acute nor chronic *H. pylori* infection appeared to affect the gastric microbial composition in mice despite changes in the stomach pH and *Helicobacter*-induced inflammation. Animal studies often aim to model human disease, and unlike with mice, the human stomach is poorly colonized by microorganisms. Therefore, it is interesting that the murine gastric microbiota, consisting largely of lactobacilli, appears to be independent of *H. pylori* infection status. This finding may prove important to studies examining the probiotic effects of lactobacillus strains present in a normal mouse stomach.

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REFERENCES

1. Aebischer, T., A. Fischer, A. Waldduck, C. Schlotelburg, M. Lindig, S. Schreiber, T. F. Meyer, S. Bereswill, and U. B. Gobel. 2006. Vaccination prevents *Helicobacter pylori*-induced alterations of the gastric flora in mice. *FEMS Immunol. Med. Microbiol.* **46**:221–229.
2. Brown, J. F., and E. Balish. 1978. Gastrointestinal microecology of BALB/c nude mice. *Appl. Environ. Microbiol.* **36**:144–159.
3. Ferrero, R. L., J.-M. Thiberge, M. Huerre, and A. Labigne. 1998. Immune responses of specific-pathogen-free mice to chronic *Helicobacter pylori* (strain SS1) infection. *Infect. Immun.* **66**:1349–1355.
4. IARC. 1994. Schistosomes, liver flukes and *Helicobacter pylori*. IARC Monogr. Eval. Carcinog. Risks Hum. **61**:177–240.
5. Kaparakis, M., K. L. Laurie, O. Wijburg, J. Pedersen, M. Pearse, I. R. van Driel, P. A. Gleeson, and R. A. Strugnell. 2006. CD4⁺ CD25⁺ regulatory T cells modulate the T-cell and antibody responses in helicobacter-infected BALB/c mice. *Infect. Immun.* **74**:3519–3529.
6. Lee, A., J. O'Rourke, M. Corazon de Ungria, B. Robertson, G. Daskalopoulos, and M. F. Dixon. 1997. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. *Gastroenterology* **112**:1386–1397.
7. Monstein, H.-J., A. Tiveljung, C. H. Kraft, K. Borch, and J. Jonasson. 2000. Profiling of bacterial flora in gastric biopsies from patients with *Helicobacter pylori*-associated gastritis and histologically normal control individuals by temperature gradient gel electrophoresis and 16S rDNA sequence analysis. *J. Med. Microbiol.* **49**:817–822.
8. Mowat, C., C. Williams, D. Gillen, M. Hossack, D. Gilmour, A. Carswell, A. Wirz, T. Preston, and K. E. L. McColl. 2000. Omeprazole, *Helicobacter pylori* status, and alterations in the intragastric milieu facilitating bacterial N-nitrosation. *Gastroenterology* **119**:339–347.
9. Nadkarni, M. A., F. E. Martin, N. A. Jacques, and N. Hunter. 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* **148**:257–266.
10. Roach, S., D. C. Savage, and G. W. Tannock. 1977. Lactobacilli isolated from the stomach of conventional mice. *Appl. Environ. Microbiol.* **33**:1197–1203.
11. Sait, L., M. Galic, R. A. Strugnell, and P. H. Janssen. 2003. Secretory antibodies do not affect the composition of bacterial microbiota in the terminal ileum of 10-week-old mice. *Appl. Environ. Microbiol.* **69**:2100–2109.
12. Sun, Y. Q., H. J. Monstein, L. E. Nilsson, F. Petersson, and K. Borch. 2003. Profiling and identification of Eubacteria in the stomach of Mongolian gerbils with and without *Helicobacter pylori* infection. *Helicobacter* **8**:149–157.
13. Sutton, P., S. J. Danon, M. Walker, L. J. Thompson, J. Wilson, T. Kosaka, and A. Lee. 2001. Post-immunisation gastritis and *Helicobacter* infection in the mouse: a long term study. *Gut* **49**:467–473.
14. Suzuki, K.-I., Y. Kokai, N. Sawada, E. Isogai, H. Isogai, and M. Mori. 2002. SS1 *Helicobacter pylori* disrupts the paracellular barrier of the gastric mucosa and leads to neutrophilic gastritis in mice. *Virchows Arch.* **440**:318–324.
15. Taylor, D. N., and M. J. Blaser. 1991. The epidemiology of *Helicobacter pylori* infection. *Epidemiol. Rev.* **13**:42–59.