Male Syrian Hamsters Experimentally Infected with Helicobacter spp. of the H. bilis Cluster Develop MALT-Associated Gastrointestinal Lymphomas

HHS Public Access

Author manuscript

Helicobacter. Author manuscript; available in PMC 2017 June 01.

Published in final edited form as:

Helicobacter. 2016 June ; 21(3): 201–217. doi:10.1111/hel.12265.

Male Syrian hamsters experimentally infected with *Helicobacter* spp. of the *H. bilis* cluster develop MALT-associated gastrointestinal lymphomas

Stephanie E. Woods[1], **Courtney Ek**[1], **Zeli Shen**, **Yan Feng**, **Zhongming Ge**, **Sureshkumar Muthupalani**, **Mark T. Whary**, and **James G. Fox**[*]

Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA 02139

Abstract

Background—Aged hamsters naturally infected with novel *Helicobacter* spp. classified in the *H. bilis* cluster develop hepatobiliary lesions and typhlocolitis.

Methods—To determine if enterohepatic *H.* spp. contribute to disease, *Helicobacter*-free hamsters were experimentally infected with *H.* spp. after suppression of intestinal bacteria by tetracycline treatment of dams and pups. After antibiotic withdrawal, weanlings were gavaged with 4 *H. bilis*-like *Helicobacter* spp. isolated from hamsters or *H. bilis* ATCC 43879 isolated from human feces, and compared to controls ($n = 7$ per group).

Results—*H. bilis* 43879 dosed hamsters were necropsied at 33 WPI due to lack of detectable infection by fecal PCR; at necropsy, 5/7 were weakly PCR positive but lacked intestinal lesions. The remaining hamsters were maintained for ~95 WPI; chronic *H.* spp. infection in hamsters (6/7) was confirmed by PCR, bacterial culture, FISH and ELISA. Hamsters had mild to moderate typhlitis, and three of the male *H.* spp. infected hamsters developed small intestinal lymphoma, in contrast to one control. Of the three lymphomas in *H.* spp. infected hamsters, one was a focal ileal MALT B cell lymphoma, while the other two were multicentric small intestinal large B cell lymphomas involving both the MALT and extra-MALT mucosal sites with lymphoepithelial lesions. The lymphoma in the control hamster was a diffuse small intestinal lymphoma with a mixed population of T and B cells.

Conclusions—Results suggest persistent *H.* spp. infection may augment risk for gastrointestinal MALT origin lymphomas. This model is consistent with *H. pylori/heilmannii* associated MALT lymphoma in humans and could be further utilized to investigate mechanisms of intestinal lymphoma development.

INTRODUCTION

The genus *Helicobacter* includes a variety of named and unnamed species (spp.) isolated from rodents, including *H. muridarum [1], H. hepaticus [2], H. bilis [3]* and *H. rodentium [4]* in mice. Murine helicobacters are of general interest, in part due to their genetic

[*]Corresponding Author, Division of Comparative Medicine, Massachusetts Institute of Technology, 77 Massachusetts Avenue, 16-825, Cambridge, MA 02139, jgfox@mit.edu, Phone: 617-253-1735, Fax: 617-258-5708 .

[1]Co-First Authors

similarities to the important human pathogen, *H. pylori*, and their use in murine models of human disease, but also because these bacteria can confound studies, even in the absence of clinical signs [5]. Mouse colonies, for example, are frequently screened for enterohepatic *Helicobacter* spp. (EHS), which are known to cause chronic hepatitis, hepatic neoplasia and inflammatory bowel disease in susceptible mouse strains [2, 6-10].

Helicobacter infections in Syrian hamsters (*Mesocricetus auratus)* are less well studied. A number of hamster *Helicobacter* spp. have been previously isolated and characterized: *H. cinaedi* and *H. mesocricetorum* from clinically normal hamsters [11-13]; *H. aurati* from hamsters with gastritis, intestinal metaplasia and inflamed ceca, including one report of gastritis-associated adenocarcinoma [14-16]; and *H. cholecystus* from gallbladders of hamsters with cholangiofibrosis and pancreatitis [17]. Based on the available literature, the same potential to interfere with research results or for use in animal model development that exists for mice is also possible for hamsters infected with *Helicobacter* spp. We have described *Helicobacter*-associated progressive, proliferative and dysplastic typhlocolitis in Syrian hamsters aged 18 to 24 months [18], and isolated *Helicobacter* spp. classified in the *H. bilis* cluster from hamsters with chronic hepatitis, hepatic dysplasia, fibrosis and biliary hyperplasia [19].

Hamsters, being permissive and large enough to sustain infection with human liver flukes, have historically been used in liver carcinogenesis studies and as a model for liver fluke-associated cholangiocarcinoma [20]. The literature ascribes the carcinogenic potential of liver flukes in part to studies conducted in hamsters; most of these studies were undertaken prior to the isolation of *Helicobacter* spp. in hamsters and its association with hepatobiliary disease [19, 20]. Background *Helicobacter*-associated pathology could be contributing to the chronic hepatic lesions attributed to liver flukes. Serologic evidence of *H. bilis* infection has been reported in Thai people with liver fluke infections [21], and an association was established between *H. bilis* and biliary cancer in two high-risk populations in Thailand and Japan [22]. Also, *H. bilis* DNA has been found in the gallbladder tissue of humans with cholecystitis who have increased risk of developing gallbladder cancer [23]. Further, authors of one study co-cultured a human bile duct cancer cell line with *H. bilis* and found that the *H. bilis*-infected cell line had enhanced NF-κB activity and production of VEGF, suggesting that the increased angiogenesis observed in cell cultures may contribute to malignancy in humans [24]. A recent study of Mexican patients with extrahepatic cholangiocarcinoma found that the presence of *H. bilis* in the biliary tract was significantly associated with common bile duct cancer, suggesting risk attributable to *H. bilis* in the absence of endemic liver fluke infection [25].

To develop a hamster model to probe the association of *H. bilis* with liver fluke-associated cholangiocarcinoma, the goal of our study was to establish a causal relationship between *Helicobacter* spp. and the gastrointestinal and hepatobiliary diseases of aged hamsters.

METHODS

Hamsters

For experimental infection of *Helicobacter*-free Syrian hamsters with *Helicobacter* spp. of the *H. bilis* cluster, three pregnant HsdHan™:AURA dams were obtained from Harlan Laboratories (Indianapolis, IN) and the pups were divided into three groups: *H.* spp. dosed (n = 7; 4 males, 3 females), control (n = 3; 2 males, 1 female), and *H. bilis* 43879 dosed (n = 7; 4 males, 3 females) hamsters (Table 1). Four months into the study, two male and two female age-matched retired breeders from Harlan Laboratories were purchased as additional controls (Table 1). HsdHan™:AURA hamsters were free of select exogenous viruses, bacterial pathogens and parasites, including *Helicobacter* and *Campylobacter* spp.

To validate serology as a screening tool for *Helicobacter* infection status in Syrian hamsters, 18 adult *Helicobacter*-free hamsters were purchased from a commercial vendor (Source A) between 2010 and 2011, and maintained in isolation from other source hamsters to serve as negative controls. Hamsters were confirmed free of *Helicobacter* spp. by PCR and bacterial culture of gastrointestinal tissues, as described below. Sera were also obtained from 26 adult Syrian hamsters submitted to our laboratory for diagnostic evaluation between 1998 and 2004 [15, 16, 19]. Of these, six (n = 3 males, 3 females) *Helicobacter*-free hamsters were obtained from Source A between 1998 and 1999, as previously reported [15, 18, 19]. Additionally, hamsters naturally infected with *Helicobacter* spp. were obtained from 2 other commercial vendors (Source B: n = 5 females & Source C: n = 5; 3 males, 2 females) and two academic institutions (Source D: n = 5; 3 males, 2 females & Source E: n = 5; 4 males, 1 female).

All hamsters were maintained in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International, and experimental use was approved by MIT's Committee on Animal Care.

Husbandry

All hamsters used for experimental infection were singly housed in polycarbonate microisolator cages with filter tops (Lab Products, Inc., Seaford, DE) on heat-treated hardwood bedding (Sanichips, PJ Murphy Inc., Montville, NJ), and provided water and diet (Prolab RMH 3000, LabDiet, St. Louis, MO) *ad libitum*. Most hamsters used for ELISA validation were also singly housed, with some younger hamsters (< 6 months of age) in compatible pairs with littermates. Macroenvironmental conditions included a 12:12 light / dark cycle and temperature maintenance at 72 ± 2°F, with 30 to 70% humidity and 10 to 15 air changes per hr.

Experimental infection with *Helicobacter* spp. of the *H. bilis* cluster

To suppress intestinal bacteria, all dams and pups, including controls, were treated with tetracycline in the drinking water (0.4 mg/mL; Ornacycline, Sentry AV) beginning four days after birth. Hamsters were acclimated to tetracycline-treated water for the first three days with non-treated water, then non-treated water was removed and only tetracycline-treated water was provided for an additional 17 days. Note that hamster pups begin to eat solid food

at 7-10 days of age and consumed water in addition to dam's milk [26]. Following a 3-day tetracycline washout period, pups were weaned and divided into experimental groups at 24 days of age.

Inoculation began on the day of weaning, with *H.* spp. dosed hamsters receiving an equal mixture of 4 *H. bilis*-like strains isolated from hamsters with identical 16SrRNA sequences (MIT 04-196, 04-199, 04-384, 08-7249; [19]), while another group of hamsters were dosed with *H. bilis* ATCC 43879 isolated from human feces. *H.* spp. and *H. bilis* ATCC 43879 were cultured on trypticase soy agar with 5% sheep blood plates (blood agar plate; BAP) for two days under microaerobic conditions, as described below. Bacteria were collected using cotton swabs and resuspended in Brucella broth with 20% glycerol; the bacterial concentration was adjusted to 1 OD600/mL (3×10^7 to 3×10^8 organisms). *H.* spp. and *H. bilis* 43879 dosed hamsters each received 0.3 mL of fresh inoculum via oral gavage, every other day for three doses; controls were sham-dosed with Brucella broth, excluding the four unmanipulated retired breeders received at a later date.

Necropsy

H. spp. dosed and control hamsters were, on average, euthanized at 96 and 94 weeks post-infection (WPI), respectively (Table 1); one *H.* spp. dosed hamster (91 WPI) and four controls (88, 92, 94, 96 WPI) were euthanized prematurely due to declining health. *H. bilis* 43879 dosed hamsters were euthanized at 33 WPI (Table 1). All hamsters were euthanized using CO_2, weighed, had body condition score (BCS) determined and underwent complete postmortem gross examination. The gastrointestinal tract (including liver, stomach, small intestine, cecum and colon), as well as lungs, spleen, bone marrow and mesenteric lymph node in select hamsters, were harvested for PCR, bacterial culture and histopathologic analysis, and cardiac blood was collected postmortem for hematologic work-up and serology for ELISA, as described below.

Screening for hematopoietic or metabolic abnormalities

Complete blood counts (CBC) were performed on 6 *H.* spp. dosed hamsters ($n = 4$ males, 2 females) and 3 controls ($n = 1$ male, 2 females), using a Hemavet 950FS analyzer (Drew Scientific, Waterbury, CT). Also, sera were sent to IDEXX Laboratories (Memphis, TN) for a chemistry panel of 21 analytes from all 7 *H.* spp. dosed hamsters ($n = 4$ males, 3 females) and 5 controls ($n = 4$ males, 2 females).

DNA extraction and *Helicobacter* spp. PCR

Fresh fecal samples were periodically collected from all *H.* spp. and *H. bilis* 43879 dosed hamsters throughout the course of study to monitor colonization status of dosed hamsters. At necropsy, feces and sections of the gastrointestinal tract (including liver, stomach, duodenum, ileum, cecum and colon) of all *H.* spp. and *H. bilis* 43879 dosed hamsters were aseptically harvested and frozen at −80°C until DNA extraction; cecal tissue, cecal contents and feces were also collected from controls, to confirm *Helicobacter*-free status at end of study. DNA was extracted from feces using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) and from tissues using the HighPure PCR Template Preparation Kit (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer's instructions.

PCR analysis was performed in a 50 μL reaction volume containing 10X Expand High Fidelity buffer with 15 mM $MgCl_2$, 0.5 mM each of *Helicobacter* genus-specific primers (C05 and C97, or C97 and C98) [23], 200 μM dNTP, 200 μg/mL bovine serum albumin (BSA) and 2.5 U Expand High Fidelity Enzyme mix (Roche Diagnostics Corporation, Indianapolis, IN). The *Helicobacter* genus-specific PCR product (C05/C97: 1200 bp; C97/C98: 400 bp) was separated electrophoretically in a 1% agarose gel, according to a previously published protocol [23]. PCR products were visualized by ethidium bromide staining.

Real-time quantitative PCR was also performed on liver and cecal DNA from all *H.* spp. and *H. bilis* 43879 dosed hamsters and 2 control ceca (n = 2 females), as previously described [27, 28].

Microbiological culture for *Helicobacter* spp

All PCR-positive cecal tissue samples of *H.* spp. and *H. bilis* 43879 dosed hamsters were homogenized in sterile culture medium, composed of Brucella broth with 20% glycerol, for microaerobic culture. The homogenate was passed through a 0.65 μm pore filter and plated on BAP. The homogenate was also plated directly on selective media: 5% sheep blood agar with cefoperazone, vancomycin and amphotericin B (CVA; Remel, Lenexa, KS). The plates were incubated at 37°C under microaerobic conditions in vented jars containing N_2, H_2 and CO_2 (80:10:10) and maintained for at least 2 weeks with periodic evaluation.

The identity of cultured organisms was confirmed as *Helicobacter* spp. by colony morphology, Gram stain, phase contrast microscopy, biochemical reactions and/or PCR with genus-specific primers, as described above.

Outer membrane preparation and ELISA for anti-*Helicobacter* IgG in serum

H. bilis (ATCC 43879) and *H.* sp. 08-7249 OMP antigen were obtained by methods previously described for preparing *H. hepaticus* antigen [29]. Briefly, *H. bilis* and *H.* sp. 08-7249 were cultured for 48 hr under microaerobic conditions. After three washes with PBS and examination for bacterial contaminants using gram stain and phase contrast microscopy, the pellets were resuspended in 1% N-octyl-beta-glucopyranoside (Sigma-Aldrich Co. LLC., St. Louis, MO) for 30 min at room temperature. Insoluble material was removed by ultracentrifugation at 100,000 *g* for 1 hr. After dialysis against PBS for 24 hr at 4°C, the supernatant protein concentration was measured by the Lowry technique (Sigma-Aldrich Co. LLC., St. Louis, MO).

For serum IgG measurement, 96-well plates were coated with 100 μL per well of 1 μg/mL *H. bilis* or *H.* sp. OMP antigen in carbonate buffer (pH 9.6) overnight at 4°C. Plates were blocked with 200 μL per well of 2% bovine serum albumin (BSA) in PBS. Serum samples were diluted 1:100 with 1% BSA in PBS, and biotinylated goat anti-hamster IgG (1:1000; Bethyl Laboratories, Inc., Montgomery, TX) was used as the secondary antibody; steps were preceded by six washes and followed by incubation at 37°C for 1 hr, with 100 μL plated per well. Following a 30 min incubation with extravidin peroxidase (1:2000; Sigma-Aldrich Co. LLC., St. Louis, MO), 100 μL of ABTS substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used for color development over 30 min in the dark at room

temperature. Optical density (OD) was detected by an ELISA plate reader at a wavelength of 405/590 nm (Dynatech MR7000, Dynatech Laboratories, Chantilly, VA). Serum IgG results are reported as mean OD values at a sample dilution of 1:100, with samples run in triplicate.

Fluorescent *in situ* hybridization

Unstained slides were prepared from paraffin-embedded blocks of cecal tissue from all *H.* spp. and *H. bilis* 43879 dosed hamsters and 1 control (female). Fluorescent *in situ* hybridization (FISH) for detection of the small subunit rRNA of *Helicobacter* spp. at a whole-cell level was performed as previously described, using a *H. bilis* ATCC 43879 probe (Cy3) and a universal bacterial probe (Eub 338-FAM) with discriminating fluorescent labels [30].

Histopathologic evaluation

Liver, stomach, small intestine, cecum and colon from all hamsters were fixed in buffered 10% formalin and processed for histology. Tissues were embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin (H&E), and evaluated by a board-certified veterinary pathologist blinded as to sample identity. Liver sections were assessed on a scale of 0 (normal) to 4 (severe) for lobular, portal and interface inflammation, and dysplasia/neoplasia. The number of lobes with >5 inflammatory foci was also used to calculate a cumulative hepatitis index score, as previously described [31]. The antrum, including pylorus, and corpus of stomach sections were independently graded on a scale of 0 (normal) to 4 (severe) for inflammation, epithelial defects, hyperplasia and dysplasia/neoplasia. Sections of corpus were also scored for oxyntic atrophy, pseudopyloric and mucous metaplasia, GHAI (gastric histologic activity index) and hyalinosis [32]. Small intestines, ceca and colons were also semi-quantitatively evaluated on a scale of 0 (normal) to 4 (severe) for histomorphological changes, including inflammation, epithelial defects, edema, epithelial or crypt atrophy, hyperplasia and epithelial dysplasia/neoplasia, and cumulative typhlitis and colitis scores determined [32].

Intestinal lymphomas, including mucosa-associated lymphoid tissue (MALT) lymphomas, were diagnosed on the basis of established WHO criteria for classifying human lymphomas [33]. Gastric and intestinal MALT tissues were evaluated for both non-neoplastic changes as well as neoplastic changes, such as necrosis, hyperplasia, atypical cell morphology, distribution and cellular pattern of expansion within MALT and surrounding tissues. The presence of lymphoepithelial lesions (LE) and basic immunohistochemical profiles (IHC; Ki67, B and T cell markers), as described earlier for both rodent and human tissues were recorded [34-38] .

Three male *H.* spp. dosed hamsters with gastrointestinal lymphoma, predominantly involving MALT, and one male control with diffuse lymphoma were further evaluated by H&E staining for evidence of multisystemic lymphoma, including by qualitative histologic assessment of lungs, spleen and bone marrow for any relevant pathology, and compared to one female control lacking gross and microscopic evidence of small intestinal lymphoma.

Immunohistochemistry of MALT lymphoma

To determine B or T cell type of small intestinal lymphomas in three *H.* spp. dosed hamsters and one control, as well as cell type of a multisystemic lymphoma in another control, gastrointestinal sections from these hamsters and 1 male lymphoma-free control were used for evaluation of immunoreactivity with the following antibodies: CD45 B220 [1:6000 dilution; low antigen retrieval (AR) pH; Abcam], CD79a (1:50 dilution; high AR pH; Novus), CD3 (1:600 dilution; high AR pH; Dako) and Ki67 (1:50 dilution; high AR pH; BD Pharmigen). Unstained slides were prepared as described above, and incubated at 60°C for 30 min, followed by deparaffinization and rehydration prior to analysis. Antigen was retrieved at a low or high pH, depending on the antibody, for 45 min at 95°C. An automated stainer (Lab Vision Autostainer 360, Thermo Fisher Scientific, Waltham, MA) was used for a 10 min Ultravision peroxidase block, a 30 min Rodent block R, a rabbit-on-rabbit (CD45 B220 for 60 min; CD3 for 30 min) or mouse-on-mouse (CD79a for 30 min; Ki67 for 60 min) secondary polymer application, and a 10 min application of diaminobenzidine. H&E counterstaining was then performed manually, as described above. Slides were dehydrated and cleared, and then coverslipped for microscopic analysis. LE lesions were highlighted by a similar immunostaining procedure described above, using cytokeratin 20 (CK20) polyclonal antibody (1:200 dilution; low AR pH; Abcam; small intestinal mucosa) and pancytokeratin AE1/AE3 antibody (1:75 dilution; low AR pH; Dako; stomach mucosa; data not shown).

Statistical analysis

Age, body weight, CBC and serum chemistry values were compared between *H.* spp. dosed hamsters and controls using a two-sample test of group means assuming equal variance (two-tailed), and reported as mean ± standard deviation (SD). Percentage data (hematocrit, red blood cell distribution width) were arcsin transformed prior to analysis. A two-sample test of group means was also used to compare ELISA OD values of *H.* spp. and *H. bilis* 43879 dosed hamsters with controls. Median BCS and histopathology scores were compared between *H.* spp. dosed hamsters and controls using a Mann-Whitney two-sample rank-sum test. *H.* spp. or *H. bilis* 43879 dosed hamsters determined by PCR to be uninfected were excluded from statistical analyses.

For ELISA validation, a serum sample was considered positive for IgG antibody to *Helicobacter* spp. if the OD measured for the sample exceeded a value equal to the mean plus 3 SD of the OD measured for Source A *Helicobacter*-free samples. Comparisons between groups were made using a two-sample test of group means assuming equal variance (two-tailed), and the sensitivity, specificity, positive and negative predictive values of the ELISAs for detecting *Helicobacter*-infected hamsters were calculated, with PCR and bacterial culture positivity required for a true positive.

Statistical analysis was performed using STATA/IC 13.0 for Mac (StataCorp; College Station, TX) and Prism Version 5.0 (GraphPad Software; La Jolla, CA), with $p<0.05$ considered statistically significant.

RESULTS

Experimental infection of hamsters with *Helicobacter* spp. of the *H. bilis* cluster

Six of 7 (86%) *H.* spp. dosed hamsters were colonized with *Helicobacter* spp., as demonstrated by PCR positivity in feces throughout the 96 week infection period (Tables 1 and 2). One male *H.* spp. dosed hamster showed a weak band until 4 WPI, but became negative; this likely indicated a low colonization level, with subsequent clearance of the organisms (Tables 1 and 2). Postmortem characterization of infection revealed robust infection in the ceca of all 6 fecal PCR-positive *H.* spp. dosed hamsters (qPCR, cecal tissue: mean: 6.1×10^4; range: 1.1×10^4 to 1.8×10^5 copies/μg DNA) (Table 2; Figure 1A). PCR-positive cecal tissue samples were also positive by bacterial culture and FISH for *Helicobacter* spp. (Figure 1D-E,G). Liver, stomach, duodenum, ileum and colon of *H.* spp. dosed hamsters were all negative for *Helicobacter* spp. by PCR, except for three hamsters (two of which had MALT lymphoma), where a total of two duodenum samples ($n = 1$ male, 1 female) and two ileum samples ($n = 1$ male, 1 female) were positive (Table 2).

The control hamsters were maintained *Helicobacter*-free throughout the duration of study as determined by negative PCR results on cecal tissue, cecal contents and feces. Control hamsters were statistically equal in age, body weight and body condition to *H.* spp. dosed hamsters (Table 1; Figure 1A).

All *H. bilis* 43879 dosed hamsters were euthanized because they were repeatedly negative for *Helicobacter* spp. by fecal PCR throughout the 33 week infection period. Feces, liver, stomach, duodenum, ileum and colon were all negative for *Helicobacter* spp. by PCR. However, cecal tissue analyzed postmortem by PCR indicated cecal colonization in 5 of 7 (71%) inoculated hamsters, at low levels by qPCR (mean: 2.2×10^2; range: 3.1×10^1 to 6.3×10^2 copies/μg DNA) (Table 1; Figure 1B). *Helicobacter* spp. PCR-positive cecal tissue samples were also positive by FISH, but negative by bacterial culture (Figure 1F).

H. spp. infected hamsters had significant antibody responses to experimental *Helicobacter* infection

For ELISA validation, all 24 ($n = 18$ purchased, 6 historically obtained) hamsters from Source A were confirmed *Helicobacter*-free by PCR and bacterial culture. All hamsters from Sources B, C, D and E were confirmed *Helicobacter*-infected by PCR and bacterial culture. *Helicobacter*-free hamsters ($n = 18$; *H. bilis* OD: 0.30 ± 0.23; *H.* sp. 08-7249 OD: 0.38 ± 0.16) had 4.7- and 4.1- fold lower mean OD values compared to *Helicobacter*-infected hamsters ($n = 20$; *H. bilis* OD: 1.42 ± 0.31; *H.* sp. 08-7249 OD: 1.56 ± 0.22), using the *H. bilis* and *H.* sp. 08-7249 OMP antigens, respectively (both $p<0.001$). The cut-off for considering hamster *Helicobacter*-positive by serology for the *H. bilis* OMP antigen was 0.99, while the cut-off for *H.* sp. 08-7249 was 0.86. ELISA using *H. bilis* OMP antigen had a sensitivity of 90%, specificity of 88%, positive predictive value of 87% and negative predictive value of 84%; there were three false positive and four false negative results. ELISA using *H.* sp. 08-7249 OMP antigen had a sensitivity of 100%, specificity of 83%, positive predictive value of 83% and negative predictive value of 100%; there were four false positive results and no false negatives.

Based on the ELISA validation experiment, *H.* sp. 08-7249 OMP antigen (1 μg/mL) was used to determine serum IgG levels against *Helicobacter* spp. for all hamsters used for experimental infection (Table 2). Consistent with robust infection, *H.* spp. infected hamsters (OD: 0.81 ± 0.56) had a significantly higher OD values than controls (OD: 0.26 ± 0.10) ($p<0.05$), whereas *H. bilis* 43879 infected hamsters (OD: 0.22 ± 0.10) were statistically identical to controls (Figure 1C). By applying the same cut-off formula (mean OD of controls + 3 SD) for considering a hamster positive for IgG antibody to *Helicobacter* spp. used above, a positive result was 0.56; 4 of six *H.* spp. infected hamsters surpassed this cut-off, while no *H. bilis* 43879 infected hamsters were positive (Table 2).

Age-matched *H.* spp. infected hamsters and uninfected controls had numerous background pathologies, including low-grade hepatitis

Gross lesions observed at necropsy of all hamsters are summarized in Table 1; no abnormalities were detected in *H. bilis* 43879 dosed hamsters euthanized at 33 WPI. Of note were hepatic cysts (Figure 2A-B) and adrenal tumors present in several of the *H.* spp. dosed hamsters and controls maintained, on average, 95 WPI. Three of 6 infected male hamsters dosed with *H.* spp. had gastrointestinal MALT lymphoma (Table 2; Figure 3A-B). Histological findings of liver and small intestine pathology are described in detail below. No statistically significant and clinically relevant differences were found in CBC and serum chemistry analytes between *H.* spp. infected hamsters and controls.

Six of 7 and 5 of 7 *H.* spp. dosed and control hamsters, respectively, had hepatic cysts, with one male *H.* spp. infected hamster presenting with multilocular hepatobiliary cystadenocarcinoma, described in more detail in Figure 2A-E (Table 1). Generally, liver sections of *H.* spp. infected hamsters and controls were characterized by few to many hepatic cysts in different lobes with sinusoidal dilatation, some with centrilobular and midzonal hepatocellular cytoplasmic clearing and swelling (glycogenic ± fatty change), occasional subcapsular hemorrhages, and the presence of pigmented macrophages and lymphoid aggregates. One male control had a multisystemic, non-GI T cell lymphoma involving the liver with multiple foci causing parenchymal effacement, in addition to affecting the mesenteric lymph nodes, kidneys, adrenal glands, pancreas and stomach; the last of which had CD3 positive metastatic foci. Hence, his liver dysplasia/neoplasia score was excluded from statistical analysis, as the pathology was not a primary hepatocellular neoplasm (Table 1). There were no statistically significant differences in liver lesion scores between *H.* spp. infected hamsters and controls, except greater interface inflammation noted in the infected hamsters ($p<0.05$) (Figure 2G). The type of inflammation observed in the livers of *H.* spp. infected hamsters appeared to be secondary to the hepatic cysts, with occasional infarctions present; however, the lesions were not typical of inflammation seen with *Helicobacter* infections in mice. *H. bilis* 43879 infected hamsters presented with none to minimal liver abnormalities, mostly consisting of fatty change and diffuse glycogenic clearing (Figure 2F-G).

Other than the lymphoma cases, most stomach and small intestine sections of *H.* spp. infected and control hamsters were within normal limits, characterized by mild, mixed

inflammation of lymphocytes, plasma cells and macrophages. *H. bilis* 43879 infected hamsters had no significant pathology in either the stomach or small intestine (Figure 3K).

Hamsters infected with *H.* spp. had a high prevalence of gastrointestinal lymphoma predominantly involving MALT

Representative gross and histopathologic findings of the small intestine of three *H.* spp. infected male hamsters with gastrointestinal MALT-predominant lymphoma (prevalence: 50%) are summarized in Figure 3. IHC using Ki67, CD45 B220 and CD79a, and CD3 as cell proliferation, B cell and T cell markers, respectively, classified all three cases of MALT lymphoma as B cell origin, with cell proliferation patterns mirroring CD45 B220 (not shown) and CD79a staining (Figure 3F-H).

The three lymphomas in the *H.* spp. infected hamsters included one focal ileal MALT lymphoma (Figure 3A,C-D), and two similar multicentric lymphomas predominantly involving MALT and extra-MALT areas (Figure 3B,E-J) (Table 2). The focal ileal MALT lymphoma was centered on a gut-associated lymphoid tissue (GALT) area that was distorted in follicular organization and was markedly enlarged in size, with expansion into lamina propria and villi. Plasma cell differentiation was also noted, with expansion of the neoplastic B cells into the surface epithelium.

The two multicentric intestinal lymphomas in the *H.* spp. infected male hamsters had similar prominent MALT centered proliferations of large neoplastic B cells with effacement of glands by abundant Ki67 positive cells (pale cytoplasm and large, irregular nucleus; monocytoid appearance) admixed with relatively quiescent intersecting T cell rich areas (Figure 3F-H). LE lesions were highlighted by CK20 labeling of remnant, partially effaced glands infiltrated with neoplastic B cells in neoplastic MALT foci (Figure 3I-J). Further, in many areas there was expansion of neoplastic B cells into the lamina propria, submucosa, muscularis, serosa and adjacent mesentery. These two cases likely represent ongoing transformation of MALT lymphoma into diffuse intestinal lymphoma. One of the 2 *H.* spp. infected hamsters with multicentric intestinal lymphoma also presented with focal MALT hyperplasia with some atypical large, blast-like cells in the pyloric duodenum that were similar to those seen in the small intestine (Table 2). Complete postmortem gross examination and histologic assessment, including of the lungs, spleen and bone marrow, of these three *H.* spp. infected hamsters revealed no evidence of multisystemic lymphoma, supporting their small intestinal origin, and likely of MALT origin.

One male control with a MALT activity score of 4.0 was histologically classified as a diffuse, small intestinal lymphoma with massive expansion of lamina propria and associated distortion of mucosal architecture by multifocal to coalescing mucosal and mural infiltrates of monomorphic small to medium sized, well-differentiated neoplastic cells, involving both the MALT and extra MALT intestinal areas and adjacent mesentery. Unlike the definitive B cell pattern of IHC staining observed with intestinal MALT-centric lymphoma from the three *H.* spp. infected male hamsters, the IHC results with the control hamster showed that the intestinal round cell infiltrates were composed of a mixed population of CD3 positive T cells and CD79a positive B cells (data not shown). There was no gross or microscopic evidence of gastrointestinal MALT lymphoma in *H. bilis* 43879 infected hamsters.

Aged hamsters with or without *H. bilis*-like *H.* spp. had comparable cecal and colonic inflammatory indices

Sections of cecum from *H.* spp. infected hamsters and controls showed mild to moderate typhlitis, and were characterized by mixed inflammation of lymphocytes, macrophages and granulocytes, epithelial defects, edema and hyperplasia (Figure 4A-D). Colon sections of these hamsters revealed minimal to no colon abnormalities, consisting mostly of mild lymphoplasmacytic inflammation. There were no statistically significant differences in cecum and colon lesion scores between *H.* spp. infected hamsters and controls, and *H. bilis* 43879 infected hamsters had no discernible colon pathology (Figure 4E-G).

DISCUSSION

This is the first long-term experimental infection of *Helicobacter*-free Syrian hamsters with *Helicobacter* spp. of the *H. bilis* cluster. Chronic *Helicobacter* infection (a mix of four hamster *H. bilis*-like helicobacters) was established in the majority of hamsters (86%; 6/7) almost two years after inoculation. Infection was demonstrated by cecal tissue PCR, bacterial culture, FISH and significant serologic IgG antibody responses. Necropsy revealed numerous gross lesions in age-matched *H.* spp. infected and *Helicobacter*-free control hamsters. Both groups had hepatic cystic disease with low-grade hepatitis and mild to moderate typhlitis. Consistent with *H. pylori* gastric MALT lymphoma in humans, male hamsters infected with *Helicobacter* spp. had a high prevalence (100%; 50% of total infected) of gastrointestinal lymphomas with a strong likelihood of origination from MALT.

Prior attempts by our group to experimentally infect hamsters with helicobacters proved unsuccessful, with one experiment consisting of three every other day doses of a hamster *Helicobacter* sp. (08-7249) via oral gavage in 5-week-old hamsters. In another experiment, 2-week-old hamsters received 5 doses of inoculum (08-7249), once a day, by either intraperitoneal injection or oral gavage. We attribute the successful infection of *H.* spp. dosed hamsters to inoculation of weanling age pups, who had received prior administration of tetracyline-treated water to suppress competing intestinal microflora. Further, the complex inoculum containing a mix of four closely related hamster *Helicobacter* spp. with identical 16SrRNA sequences may have contributed to the successful experimental *Helicobacter* spp. infection. This approach was used for the first successful colonization of *H. pylori* in mice [39].

Hamsters inoculated with a human strain of *H. bilis* (ATCC 43879) appeared uninfected by periodic fecal PCR for *Helicobacter* spp.; this lack of detectable infection caused us to set an earlier endpoint for this group. Postmortem cecal tissue PCR and FISH revealed that 71% (5/7) of 8.5-month-old *H. bilis* 43879 dosed hamsters were in fact colonized. Because infection was not demonstrated until postmortem, no age-matched controls were available for statistical comparisons. PCR is highly sensitive and specific; however, *Helicobacter* spp. DNA may not be detectable in the feces, depending on the site of colonization. In this study, serology was not useful in determining the infection status of *H. bilis* 43879 dosed hamsters prior to euthanasia. We suspect that *H. bilis* 4387, a human isolate (previously classified as *Flexispira rappini*) is not adapted to colonize hamsters, and therefore does not elicit a pathologic/immunologic response. ELISA OD values were low, consistent with low levels of

infection not eliciting a robust immune response, compared to robust serum IgG responses in 4 of six hamsters experimentally infected with hamster *Helicobacter* spp.

ELISA is a sensitive, but not specific, tool to screen research mice for *Helicobacter* infection [40, 41], but this method has not been developed for screening hamster colonies. Laboratory hamsters are often colonized by multiple gastric and/or enterohepatic *Helicobacter* spp., many of which have not been fully characterized [11, 12, 14-17]. Our ELISA validation experiment documents the utility of ELISA for robust discrimination between naturally *Helicobacter*-infected and –free hamsters, providing a high sensitivity and a high negative predictive value; differentiation between *Helicobacter* spp. was not attempted. In murine studies, identification of helicobacters at the species level by ELISA is not reliable [40, 41]. Further studies would be needed to determine the cross-reactivity of immunogenic proteins between different species of hamster helicobacters. Serologic testing of hamsters for antibodies to *Helicobacter* spp. should prove useful in future experimental infection studies, as well as for detection of naturally occurring infections in laboratory colonies, the latter of which necessitates a rapid and relatively inexpensive, pre-mortem screen.

We were unable to prove our hypothesis that the underlying gastrointestinal and hepatobiliary lesions reported in aged hamsters are due to infection with *Helicobacter* spp. Despite no statistically distinguishable differences in intestinal histopathology in *H.* spp. infected hamsters, compared to controls, aging-associated changes may have been augmented by *Helicobacter* spp. infection. While both aged *H.* spp. infected and control hamsters had pathologies often attributed to spontaneous disease/aging, there were trends of increased small intestinal inflammation, epithelial defects and atrophy, and MALT activity, as well as cecal epithelial defects, edema and typhlitis in *H.* spp. infected hamsters; these trends were most apparent when the low lesion scores of younger *H. bilis* 43879 infected hamsters were also qualitatively considered. While a lack of lesions attributable to helicobacters in hamsters was consistent with infections in outbred and most inbred mice, we expect that a larger study would distinguish true differences in disease resulting from *Helicobacter* spp. infection.

Because of severe hepatic disease from cysts commonly found in aged hamsters [42, 43], liver lesions in *H.* spp. infected and control hamsters were difficult to interpret. Significant hepatic interface inflammation was present in *Helicobacter*-infected hamsters, as well as trends of increased lobular inflammation, the number of lobes with >5 inflammatory foci and hepatitis, even though all livers were negative for *Helicobacter* spp. by PCR. Coinfection of hamsters with liver flukes may facilitate *Helicobacter* spp. infection in the normally bacteriostatic hepatobiliary system, and thus favor more pronounced infection-related liver disease. Interestingly, a study in rats infected with *Fasciola hepatica* found that bacteria with *H. bilis*-like morphology were present only in the common bile ducts of liver fluke-infected rats [44]. The authors speculated that the helminth infection caused changes in the biliary environment that encouraged the colonization and persistence of the *H. bilis*-like bacteria [44].

Among the various pathologies detected in age-matched hamsters in this study, gastrointestinal MALT B cell lymphoma was observed in 100% (3/3) of *H.* spp. infected

male hamsters [50% (3/6) of total infected], but not in controls. Horizontally transmitted malignant lymphomas associated with hamster polyomavirus (HaPV) have historically been reported in hamster colonies; however, the characteristics of this infectious malignancy do not resemble those found in our study [45-50]. Further, more recent studies documenting spontaneous neoplasms in control Syrian hamsters used in both short- and long-term carcinogenesis studies [51] and in pet hamsters [52] did not mention MALT lymphoma, as it is very likely that in most instances hamster lymphomas are part of a multisystemic process or even if within the intestine, would often present with both MALT and extra-MALT intestinal neoplastic foci. Unlike gastric MALT lymphoma, which usually presents with more focal lesions, MALT lymphoma in the small intestine of humans is frequently multicentric and often involves both the MALT or GALT areas and other mucosal segments, especially once transformed into a diffuse type; 2 of the three gastrointestinal lymphomas likely of MALT origin documented in this study resemble the latter [34-36].

MALT lymphomas are strongly associated with gastric *Helicobacter* infections in humans, with early antibiotic treatment eradicating *H. pylori* in ~80% of cases [53-57]. Animal modeling satisfied Koch's postulates, demonstrating a causative effect of *Helicobacter* spp. in gastric MALT lymphoma development in mice infected with *H. felis [58]*, and with *H. pylori* infection in gerbils [59]. Also, *Campylobacter jejuni* has been linked to a form of lymphoma in humans, referred to as immunoproliferative small intestinal disease [60], and *H. heilmannii* can cause MALT lymphoma in humans and experimentally produces MALT lymphoma in mice [61-64]. Unlike with *H. heilmannii* infection in humans where inflammation and endothelial lesions precede MALT lymphoma [62] and with *H. bilis*-induced chronic active hepatitis leading to lymphoid organ neogenesis in mice [65], the high prevalence of MALT lymphoma in the absence of severe inflammation in our *Helicobacter*-infected hamsters is also noted in *H.* spp.-infected mice; these later examples may be due in part to a more indirect mechanism of lymphomagenesis [53]. Studies in our laboratory suggest *H. bilis* may be unique among EHS, inducing gastrointestinal lymphoid hyperplasia in the absence of overt inflammatory lesions in infected gnotobiotic Swiss Webster mice [66]. Changes in immune responses or immune parameters can affect lymphocyte lineage and be initiated by different bacterial compositions, or a single species or population of bacteria [67-72]; an altered immune system may then affect lymphoma development [53]. Hamster *H. bilis*-like helicobacters as antigens could stimulate chronic proliferation of immune cells, as *H. pylori* is known to do, leading to B cell expansion [53, 73, 74]. Recent studies investigating multi-hit, B cell lymphomagenesis in a mouse model of follicular lymphoma suggests that reentry of memory B cells to germinal centers and preferential clonal expansion, among other factors, may contribute to early dissemination and progression [75]. Unfortunately, we are unable to demonstrate B cell clonality in hamster lymphoid tissues due to unavailability of antibodies specific for hamster immunoglobulins, necessary for determining the ratio of kappa to lambda chains.

To more fully elucidate mechanisms of lymphomagenesis in both hamsters and humans infected with *Helicobacter* spp., future studies should prioritize exploring the association between MALT lymphoma and hamster *H. bilis*-like *Helicobacter* spp. Our study suggests that persistent *Helicobacter* infection may augment risk for MALT lymphoma and aging-associated changes, such as typhlitis, in Syrian hamsters. Importantly, with the availability of

Helicobacter-free hamsters, coupled with experimental infection of hamsters with *Helicobacter* spp., studies with liver flukes can now probe whether *H. bilis* plays a role in hepatobiliary cancer.

DECLARATION OF FUNDING INTERESTS &ACKNOWLEDGMENTS

This work was supported in part by NIH training grant T32-OD10978-26, R01-OD011141, and P30-ES002109 to JGF. We would like to thank Joanna Richards for providing technical assistance and expertise for immunohistochemistry of MALT lymphoma and Alyssa Terestre for her assistance in manuscript preparation.

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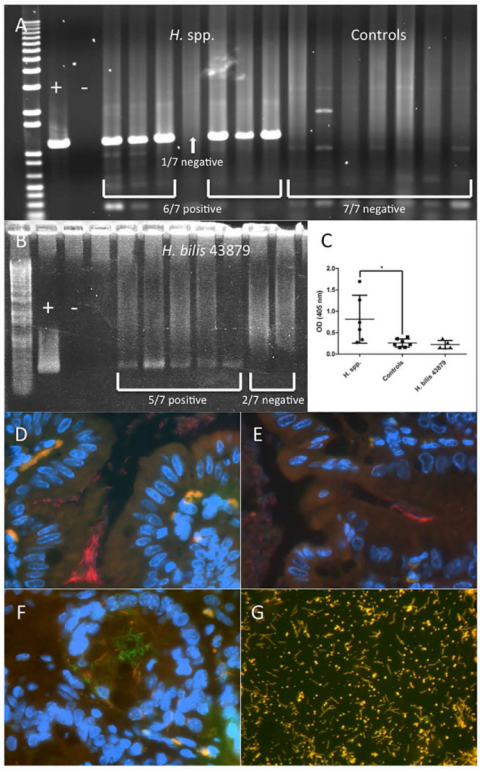

Figure 1.

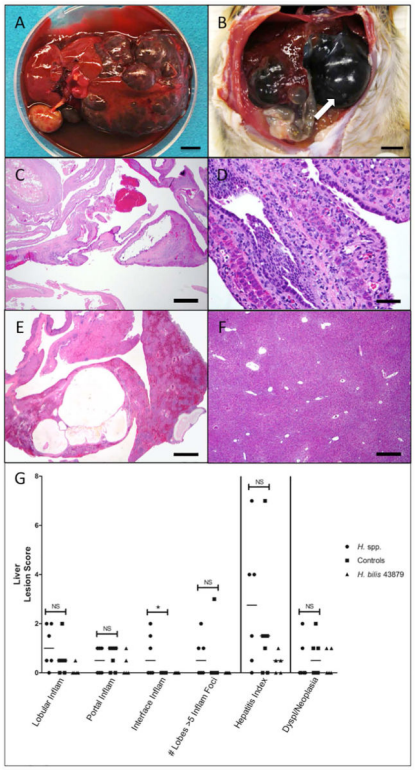

Figure 2.

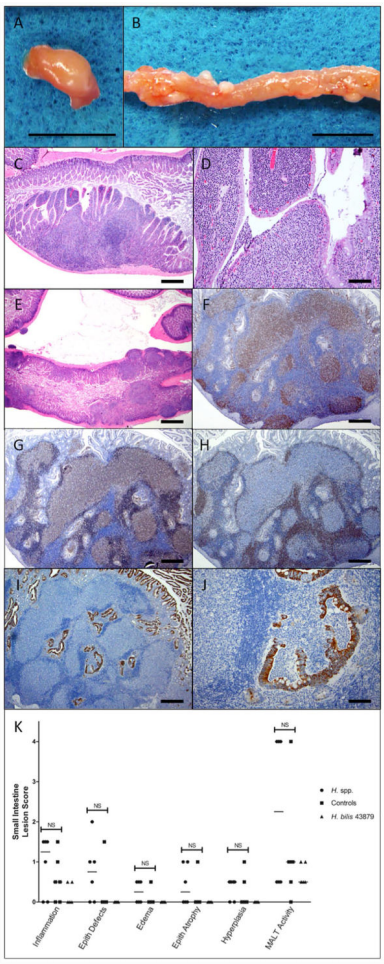

Figure 3.

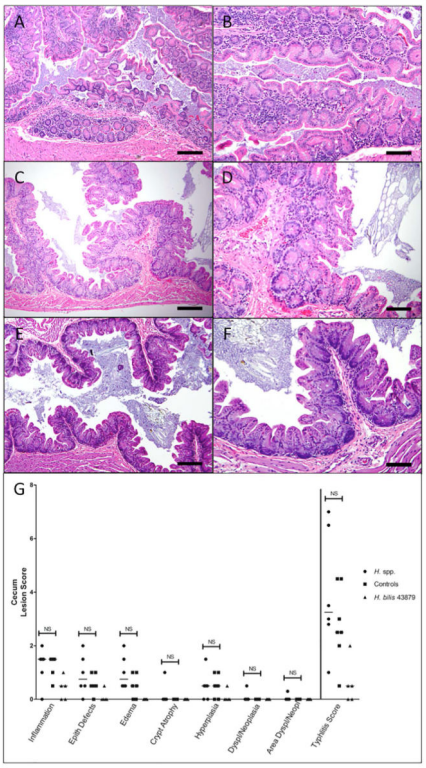

Figure 4.

Table 1

Population profile of Syrian hamsters experimentally infected with *Helicobacter* spp. of the *H. bilis* cluster

Group	*n*	[a] Sex	Infection Prevalence	Post-Infection (weeks)		Age (months)		Body Weight (grams)		Body Condition Score		[b] Pathologies
				Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Median	Range	
***H.* spp.**	7	4M, 3F	86%	96.1 ± 2.3	91 - 97	22.9 ± 0.4	22.0 - 23.0	178.2 ± 39.9	130.0 - 235.0	2.5	2.0 - 4.0	*n* = 6 HC, 4 ADT, 3 MALTL, 1 OC, 1 HCAC, 1 ATT, 1 SH
Infected	6	3M, 3F		96.0 ± 2.4	91 - 97	22.8 ± 0.4	22.0 - 23.0	184.3 ± 39.9	130.0 - 235.0	2.5	2.0 - 4.0	*n* = 5 HC, 3 ADT, 3 MALTL, 1 OC, 1 HCAC, 1 ATT, 1 SH
Non-infected	1	1M		97.0	ND	23.0	ND	141.5	ND	2.0	ND	*n* = 1 HC, 1 ADT
Controls	7	4M, 3F	0%	94.4 ± 3.4	88 - 97	22.5 ± 0.8	21.0 - 23.0	170.6 ± 24.5	125.0 - 194.0	2.5	2.0 - 3.0	*n* = 6 HC, 2 ADT, 2 OC, 1 MTL, 1 DL, 1 OSCT
***H. bilis* 43879**	7	4M, 3F	71%	33.0 ± 0.0	ND	8.5 ± 0.0	ND	145.3 ± 15.6	127.1 - 69.9	2.5	2.5 - 3.0	ND
Infected	5	2M, 3F		33.0 ± 0.0	ND	8.5 ± 0.0	ND	144.2 ± 11.3	127.1 - 155.4	2.5	ND	
Non-infected	2	2M		33.0 ± 0.0	ND	8.5 ± 0.0	ND	148.3 ± 30.5	126.7 - 169.9	2.8	2.5 - 3.0	

[a] M represents males and F represents females.

[b] HC = hepatic cysts; ADT = adrenal tumor; MALTL = gastrointestinal lymphoma, predominantly involving mucosa-associated lymphoid tissue; OC = ovarian cysts; HCAC = multilocular hepatobiliary cystadenocarcinoma; ATT = atrial thrombosis; SH = splenic hemangiosarcoma; MTL = multisystemic T cell lymphoma; DL = diffuse lymphoma; OSCT = ovarian sex cord tumor.

Table 2

Summary of experimental infection of hamsters dosed with *H. bilis*-like hamster *Helicobacter* spp., highlighting three cases of gastrointestinal lymphoma likely of MALT origin

ID	[a] Sex	*Helicobacter* spp. PCR Results							Cecal Tissue qPCR (copies/μg DNA)	ELISA OD Values	[b] B Cell GI MALT Lymphoma
		Liver	Stomach	Duodenum	Ileum	Cecum	Colon	Feces			
1	F	Neg	Neg	**Pos**	**Pos**	**Pos**	Neg	**Pos**	2.7×10^4	**1.70**	No
2	F	Neg	Neg	Neg	Neg	**Pos**	Neg	**Pos**	2.1×10^4	0.34	No
3	M	Neg	Neg	**Pos**	Neg	**Pos**	Neg	**Pos**	1.8×10^5	0.27	**Yes**; focal ileal
4	M	Neg	Neg	Neg	Neg	Neg	Neg	Neg	3.3×10^1	0.92	No
5	M	Neg	Neg	Neg	**Pos**	**Pos**	Neg	**Pos**	1.3×10^4	**0.57**	**Yes**; multicentric SI, stomach
6	M	Neg	Neg	Neg	Neg	**Pos**	Neg	**Pos**	1.1×10^4	**0.74**	**Yes**; multicentric SI
7	F	Neg	Neg	Neg	Neg	**Pos**	Neg	**Pos**	1.1×10^5	**1.27**	No

[a] M represents male and F represents female.

[b] GI = gastrointestinal; MALT = mucosa-associated lymphoid tissue; SI = small intestinal.