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PCR Detection of Colonization by *Helicobacter pylori* in Conventional, Euthymic Mice Based on the 16S Ribosomal Gene Sequence

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Many animal models of *Helicobacter* infection have been described, including infection in rhesus monkeys, ferrets, gnotobiotic piglets, and mice. These animal models utilize a combination of detection methods, including culture, urease testing, and histopathology, all of which may be unreliable, insensitive, or labor-intensive. Development of new animal models of *Helicobacter pylori* requires new methods of detection with increased sensitivity and specificity. We have developed sensitive and specific PCR primers based on the 16S ribosomal gene sequence of *H. pylori*. The primers detected single-copy 16S DNA representing 0.2 cell of pure *H. pylori* (2 cells in the presence of mouse stomach mucosal DNA) and did not cross-react with closely related bacteria. We were able to detect colonization by *H. pylori* in conventional, euthymic, outbred mice up to 4 weeks postinoculation with a high percentage of isolates tested. One isolate of *H. pylori* was detected by PCR in 100% of the mice at 6 months and 60% of the mice 1 year after inoculation. Approximately 10^3 to 10^4 *H. pylori* cells per stomach were detected by utilizing this PCR methodology semiquantitatively. These primers and PCR methodology have facilitated detection of *H. pylori* colonization in conventional, euthymic mice, colonization which may not have been detectable by other methods.

The bacterium *Helicobacter pylori* is a gram-negative, microaerophilic, spiral rod with polar flagella and a high level of urease activity (38). *H. pylori* has recently been implicated in the pathogenesis of type B gastritis and also duodenal ulcers (1, 5–7, 18, 29, 30, 40, 41). A number of animal models have been developed to study the bacterium, including those developed with the closely related bacteria *Helicobacter mustelae* and *Helicobacter felis*. These include models of *H. pylori* in nonhuman primates (2, 12, 13, 19) and gnotobiotic piglets (14, 25, 35) and the *H. mustelae* ferret (16) and *H. felis* mouse (10, 26) models. There have been numerous attempts to establish an *H. pylori* infection in mice (8, 17, 21, 23, 24). Most recently, new models of infection with human *H. pylori* isolates, utilizing germfree (GF) athymic and euthymic mice (22) and specific-pathogen-free mice, have been described (28). Detection of colonization in these models relies on culturing the organism from stomachs of challenged mice and also assessing gastric cellular infiltration histopathologically. Karita and colleagues detected colonization of *H. pylori* in the GF athymic and euthymic mice up to 10 weeks after inoculation, but only temporary (2 weeks) colonization was achieved in conventional euthymic mice (22). Marchetti and colleagues were able to detect colonization and gastric pathology in specific-pathogen-free mice up to 8 weeks following challenge with fresh clinical isolates (28).

Most currently available animal models of *Helicobacter* spp. utilize culture and/or urease testing, along with histopathology, to assess colonization following challenge and then reduction or eradication of the organism following vaccine or therapeutic regimens (9–11, 22–24, 28). With all of these methods, there are issues related to sensitivity and/or specificity which directly affect model detection limits and reliability. Culture of fastidious organisms is always a challenge with regard to the proper media, conditions, and interfering contaminants which may inhibit the growth of *Helicobacter* spp. in the conditions required. Pathology based on histological screening of a large organ such as the stomach with an infection which has random sites of localization can be a problem, especially when the tissue burden of the infecting organism is low. Additionally, the pathological condition observed may be nonspecific in terms of causative agent. Urease testing can be highly specific and effective (33, 39), especially in the absence of other microflora as in the human stomach or GF animals. However, urea broth testing is not extremely sensitive, and in rodent models there can be contaminating microflora which may also be urease producers and lead to false-positive results (44).

To further facilitate study of the organism and to develop new therapeutics or vaccines, there remains a need for small animal models of *H. pylori* infection with more sensitive and reliable methods of detection. Conventional animal models would be preferred over the use of GF animals for ease of animal manipulation and cost. In this paper, we describe detection of long-term colonization by *H. pylori* in conventional, viral antibody-free (VAF) euthymic mice by PCR amplification with a primer derived from the 16S rRNA DNA sequence of *H. pylori*. The 16S rRNA sequences are highly conserved in most bacterial species, and molecular rRNA fingerprinting is a widely utilized method of identification of bacteria to species level (27, 31). Recently, evidence has been presented to show that the DNA encoding *H. pylori* 16S rRNA is highly conserved, with two to three copies of the gene found per chromosome (27, 37) and variation in sequence of only 0.2 to 0.5% between isolates examined (15). PCR is a highly sensitive technique for detection of bacterial organisms, and recently, use of PCR based on either the *ureA* structural gene (43) or the 16S

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rRNA gene (20, 42) has been described for potential use in the diagnosis of *H. pylori* infections in patients. Therefore, a PCR primer based on the 16S rRNA gene sequence of *H. pylori* lends itself to a great degree of both specificity and sensitivity when designed properly and can be of great utility in the development of new animal models. The method of detection described in this paper has increased sensitivity over other methods utilized in animal models previously described and will facilitate further development of conventional mouse models of *Helicobacter* spp. These models may be employed for development of both new therapeutics and vaccines prior to analysis in larger animal models or human clinical trials.

MATERIALS AND METHODS

Animals. VAF and GF Swiss-Webster and VAF DBA/2 mice were obtained from Taconic Laboratories (Germantown, N.Y.). VAF CD-1 mice were obtained from Charles River Laboratories (Wilmington, Mass.). Transgenic, CD4-deficient and SCID mice were bred at our own facility at Merck and Co. Mice used for all studies were 8 to 12 weeks old at the initiation of studies and were housed in sterile microisolator cages with sterilized water and mouse chow ad libitum. Additionally, GF mice were maintained and manipulated, using sterile GF procedures, in a laminar flow hood with all surfaces sanitized with Clidox. Cages for GF mice were autoclaved in sterile wrap, and water was autoclaved and filter sterilized prior to use. Anti-Gr1 monoclonal antibody was purified from ascites fluid generated in pristane-primed Swiss outbred nu/nu mice (Taconic) by injection with RB6-8C5 hybridoma cells, a generous gift from Robert Coffmann and DNAX Corp., Palo Alto, Calif.

All animal procedures were performed in accordance with the highest standards for the humane handling, care, and treatment of research animals and were preapproved by the Merck Institutional Animal Care and Use Committee. The care and use of research animals at Merck meet or exceed all applicable local, national, and international laws and regulations.

***H. pylori*.** Human clinical isolates of *H. pylori* used in these studies were obtained from various geographical locations (CL number indicates Merck clinical culture collection) and from the American Type Culture Collection (Rockville, Md.). Bacteria were cultured on HP medium consisting of brain heart infusion agar supplemented with 5% heat-inactivated fetal bovine serum, 5% lysed horse blood, 5 mg of amphotericin B (Fungizone; E. R. Squibb, Princeton, N.J.) per liter, 5 mg of polymyxin B (Sigma Chemical Co., St. Louis, Mo.) per liter, and 5 mg of vancomycin (Sigma) per liter. Initially, all isolates were grown in batch culture, aliquoted, and frozen to provide a consistent stock of similar passage for each isolate used in all studies. Bacteria were grown on HP plates for 4 to 6 days at 37°C in microaerophilic conditions in a GasPak jar with the CampyPak Plus system (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, Md.). Bacteria were harvested from agar surfaces with sterile cotton tip swabs and resuspended in sterile saline. For challenge inoculum, the optical density of a 1:10 dilution of the inoculum was read at 660 nm. Readings of between 0.075 and 0.200 were used for challenge. Sample plating of inoculum from various optical density readings yielded 2×10^7 to 2×10^8 CFU/ml. For PCRs, bacterial cell numbers were quantitated by hemocytometer.

Urease assay. One hundred microliters of purified *H. pylori*, serially diluted in saline, was added to 900 μl of urea broth (BBL) in a test tube. Samples were then incubated aerobically at 37°C. Samples were read for color change both visually and spectrophotometrically (optical density at 545 nm) following 1, 6, and 24 h of incubation.

Experimental design. Mice were challenged orally with 0.5 ml of inoculum, prepared as described above, by gavage twice within a 1-week period (at least 1 day separating each challenge). To assess infectivity, mice were sacrificed by inhalation of CO_2 at various times postchallenge and stomachs were removed by aseptic techniques. Stomachs were then cut longitudinally, and the stomach contents were washed away by rinsing with sterile deionized H_2O. The stomach mucosa was then separated from the stomach lining tissue by gently scraping the mucosa with sterile glass microscope slides. For culture, the mucosal scrapings were spread onto HP medium, which had been preequilibrated to microaerophilic conditions, and the plates were immediately placed into a microaerophilic environment for incubation. For analysis by PCR, mucosa samples were placed in TNE (Tris-sodium chloride-EDTA) buffer and stored on ice or frozen until DNA extraction.

DNA primers for PCR. The HP primers for PCR amplification were constructed on the basis of the DNA sequence of *H. pylori* encoding the 16S rRNA gene, found in the gene bank available on the GCG Package of the Sequence Analysis Software Package Version 7.3 (Genetics Computer Group, licensed to Merck and Co., Inc.). A region of homology for six isolates of *H. pylori* listed in the gene bank (accession numbers U00679, U01328, U01329, U01330, U01331, and U01332) differing from the sequences listed for *H. felis* (M57398), *H. muridarum* (M80205), and *Campylobacter* sp. (L04315) was used for primer design. This sequence region spanned bases 793 to 1252 of the DNA sequence for *H. pylori* and resulted in a PCR product of 459 bp. The upstream primer was composed of 18 bases, bases 793 to 811, with the sequence 5′-TTG GAG GGC TTA GTC TCT-3′. The downstream primer was composed of 20 bases, bases 1252 to 1232, with the sequence 5′-AAG ATT GGC TCC ACT TCA CA-3′.

To construct an internal control template for use in the PCRs, a two-step strategy was used. First, the 495-bp PCR product was cloned into a multicopy plasmid. Subsequently, an internal restriction fragment of 237 bp, conveniently flanked by *Sty*I sites, was deleted from within the cloned *H. pylori* DNA to create a template with perfect homology to the HP primers but from which a much shorter sequence would be amplified with those primers. The 459-bp PCR-amplified *H. pylori* DNA fragment was purified by using a Geneclean kit (Bio 101, Inc., La Jolla, Calif.) and ligated with T4 DNA ligase (GIBCO BRL, Gaithersburg, Md.) into the *Eco*RV site of plasmid pBluescript II SK^+ (Stratagene Co., La Jolla, Calif.), which confers ampicillin resistance and encodes the *lacZ*α peptide. The recombinant plasmids were transformed into *Escherichia coli* DH5α (GIBCO BRL). Ampicillin-resistant transformants were selected on Luria broth plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and plasmids carrying inserted DNA were identified as giving white colonies. Plasmids were extracted from selected colonies with the Qiagen plasmid purification kit (Qiagen Inc., Chatsworth, Calif.) and cut with *Sty*I restriction endonuclease (New England BioLabs, Beverly, Mass.), which removed an internal 237-bp DNA fragment from the *H. pylori* DNA insertion. The remaining DNA was recircularized with T4 DNA ligase and transformed into strain DH5α, and colonies were selected as ampicillin resistant. Approximately 90% of such transformants yielded the desired 222-bp fragment when amplified in PCRs with the HP primers. One was selected, and plasmid DNA was extracted for use as the internal control template.

DNA extraction. The stomach mucosa suspension in TNE buffer was centrifuged for 3 min at 12,000 rpm. The supernatant was removed, and the cell pellet was resuspended in 570 μl of TNE containing 1% Triton X-100 (Sigma) and 0.5 μg of lysozyme (Sigma) per ml. Samples were then incubated at 37°C for 30 min. Next, 1 μg of proteinase K (Boehringer GmbH, Mannheim, Germany) per ml was added, and the mixture was incubated at 65°C for 2 h or at 37°C overnight. The digest was mixed with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and then centrifuged at $10,000 \times g$ for 6 min. The top aqueous layer was removed, and a second extraction with phenol-chloroform-isoamyl alcohol was performed. The aqueous layer was then mixed with an equal volume of chloroform-isoamyl alcohol (24:1) and processed as in the previous two extractions. DNA was precipitated by adding a 1/10 volume of 3 M sodium acetate and 2 volumes of absolute ethanol and placing on dry ice for 20 min. DNA was pelleted by centrifugation as described above and rinsed with 70% ethanol. The pellet was dried by speed vacuum and resuspended in 100 μl of 0.1× TE (1× TE is 10 mM Tris [pH 7.4], 0.1 mM EDTA). Samples were stored at 4°C until the PCR was run.

PCR analysis. Master reaction mixtures were prepared in a laminar flow biological safety cabinet (NuAire Inc., Plymouth, Minn.) with pipettes reserved specifically for this procedure, filter pipette tips (Fisher), and single-use aliquots of reaction components. Each master mixture contained reactants for 45 sample reactions and was made in a 1.5-ml microcentrifuge tube (Fisher). To each master mix was added 826.9 μl of deionized H_2O, 112.5 μl of 10× *Taq* buffer (Stratagene), 45 μl of deoxynucleoside triphosphate (5 mM), 22.5 μl of each primer (25 to 50 μM), and 5.6 μl of *Taq* polymerase (5 U/ml; Stratagene). The master mixtures were aliquoted at 23 μl per reaction into 200-μl PCR tubes and placed in a 96-well tray. The volume of each reaction mixture for PCR was brought up to 25 μl by adding 2 μl of templates (typically, ~2 μg of extracted DNA). The tray was briefly centrifuged to mix reactants and then placed in a Perkin-Elmer 9600 System thermal cycler (Perkin-Elmer, Norwalk, Conn.). DNA was amplified for 35 cycles of 15 s at 94°C, 30 s at 55°C, and 1 min at 72°C. The last cycle had the final 72°C elongation increased to 10 min. Positive and negative control reactions were performed with each amplification. Control templates in each run consisted of deionized H_2O, *H. pylori* DNA corresponding to 1, 10, and 100 cells, and mouse mucosal tissue DNA (2 μg). The PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide incorporation and visualized under UV light. Detection of a PCR product was scored as colonization, while absence of a product was scored as PCR negative.

Quantitation of tissue load. DNA extracted from adjusted cell numbers of *H. pylori* was used to set up a standard curve in a checkerboard orientation. Each reaction tube contained control (uninfected) stomach mucosal DNA (2 μg) corresponding to the level of a normal DNA preparation of mouse stomach mucosa for PCR runs, and then a titration of the *H. pylori* DNA was added to the reaction mixtures. For each level (titration) of *H. pylori* DNA, reaction mixtures were set up with titrated molecule levels of control DNA. The PCR amplification was run, and reactions which resulted in *H. pylori* and control DNA PCR products of equal densities when visualized on ethidium bromide-agarose gels were examined.

For analysis of unknowns, DNA from infected stomach mucosal samples was aliquoted into reaction mixture tubes for PCR (2 μg of DNA per reaction tube). Titrations of the control DNA, 0.5 $\log_{10}$ dilution from 10^3 to 10 molecules, were then added to each stomach DNA aliquot reaction tube. These reactions were placed in the same amplification run as the standard curve described above to ensure that no variation due to reactants or conditions occurred. The reaction mixtures for each stomach mucosa sample that gave equivalent PCR products within the linear product range for control DNA reactions were selected. Results

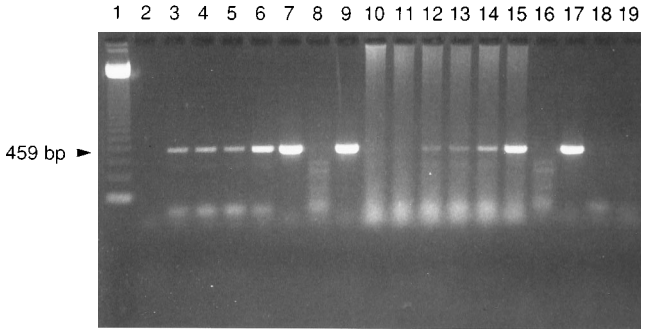

FIG. 1. PCR products from *H. pylori* (CL5173; pure culture and in tissue), *H. felis*, and *H. muridarum*. Lane 1, DNA ladder; lanes 2 and 8, deionized H_2O; lanes 3 to 7, PCR products from pure culture of *H. pylori* DNA representing 0.2 cell or approximately a single copy of the 16S rRNA gene (lane 3), 2 cells (lane 4), 20 cells (lane 5), 200 cells (lane 6), and 2,000 cells (lanes 7 and 9); lanes 10 and 16, PCR product from 2 μg of Swiss-Webster mouse stomach mucosa tissue alone; lanes 11 to 15, PCR products from *H. pylori* cells spiked into 2 μg of stomach mucosal tissue and representing DNA from 0.2 (lane 11), 2 (lane 12), 20 (lane 13), 200 (lane 14), and 2,000 (lane 15) cells; lanes 17 to 19, PCR products from 2,000 cells of pure culture of *H. pylori* (lane 17), *H. felis* (lane 18), and *H. muridarum* (lane 19).

for tissue load of *H. pylori* in unknown samples were then determined by comparison with the standard curve. The number of *H. pylori* cells per stomach was determined as the number of *H. pylori* cells which produced equivalent product in the standard curve reaction with the control molecule level which had resulted in a product of density equal to that of the unknown aliquot, multiplied by the dilution factor of unknown tissue DNA tested.

RESULTS

Sensitivity and specificity of PCR assay. A PCR amplification assay was performed to assess the sensitivity and specificity of the PCR methodology (Fig. 1). For this assay, DNA was purified from *H. pylori* CL5173, *H. felis* (a generous gift from James Fox), and *H. muridarum* ATCC 49282. *H. pylori* DNA corresponding to 0.2 (approximately a single copy of the 16S gene), 2, 20, 200, or 2,000 cells was added to reaction mixtures for PCR either alone or in the presence of mouse stomach mucosal DNA preparations. The amount of stomach mucosal DNA added to each reaction was based on the amount of tissue which would be present in a typical mouse sample and was held constant in each reaction mixture, with only the amount of *H. pylori* DNA added to each mixture varying. As shown in lanes 8 and 16, stomach mucosal DNA alone did not lead to formation of a nonspecific PCR product in the reaction. Lanes 3 through 7 show the PCR products generated by the addition of DNA equivalent to increasing *H. pylori* cell numbers, without the presence of stomach tissue DNA, and the assay detected DNA representing a single copy of the 16S gene (lane 3) or less than a single cell. When the *H. pylori* DNA was combined with stomach mucosal DNA to simulate the infected stomach DNA preparations, there was a 1-log reduction in the sensitivity of the reaction (lanes 11 through 15). The reaction in the presence of stomach mucosal DNA detected as few as 2 *H. pylori* cells (lane 12), or approximately 20 copies of the gene, but did not amplify the single-gene-copy DNA (lane 11), as was detected with *H. pylori* DNA alone (lane 3). The primers did not amplify the DNA representing 2,000 cells of either *H. felis* (lane 18) or *H. muridarum* (lane 19). Additionally, the primers did not amplify DNA isolated from *Campylobacter jejuni*, *E. coli*, and *Proteus vulgaris* (data not shown). The limit of detection of *H. pylori* in the presence of stomach mucosal tissue DNA reduced the minimum colonization detectable to 100 organisms per stomach on the basis of analysis of 1/50 of the diluted DNA sample.

The PCR amplification reaction was able to detect *H. pylori* obtained from various sources (Fig. 2), including pure culture of *H. pylori* CL5173 (lane 2), or when spiked into stomach mucosal tissue (lane 4). Additionally, the primers amplified DNA from an *H. pylori* isolate reisolated, by culture, from the stomachs of GF Swiss-Webster mice at 15 min (lane 5), 1 week (lane 6), or 5 weeks (lane 7) following oral inoculation. The primers also amplified DNA purified from stomach mucosa collected from VAF Swiss mice 4 weeks after oral inoculation with *H. pylori* CL5173 (lanes 9, 10, and 11).

Urease assay sensitivity was tested with a pure culture of *H. pylori* CL3501. Similar assay results were obtained both visually and spectrophotometrically. At 1 h the assay could detect 10^7 *H. pylori* cells and after 6 h of incubation it could detect as few as 10^6 *H. pylori* cells. Reading the assay at 24 h increased the sensitivity to 10^4 cells initially seeded in the reaction mixture. The same results were obtained in a microtiter urea assay and are in agreement with recently published limits of detection (44).

Infectivity of *H. pylori* in mouse strains. Table 1 shows the results of inoculation of various mouse strains with *H. pylori*. Both inbred and outbred strains of conventional mice were assayed 4 weeks after oral inoculation with *H. pylori* (CL3498). All mouse strains were found to be 100% colonized with this isolate when assayed by PCR. Unchallenged mice, assayed from each lot of mice received from the supplier, were consistently negative in the PCR assay.

Time course of infectivity of *H. pylori* isolates in Swiss mice. On the basis of colonization results with the mouse strains, Swiss-Webster mice, inexpensive and readily available, were selected for further studies of *H. pylori* colonization. Table 2 shows the results of PCR analysis of stomach mucosa at various times following inoculation with different clinical isolates of *H. pylori*. At 1 week following oral inoculation of mice, 8 of 11 isolates tested showed greater than 80% colonization by PCR analysis. The colonization by 23 isolates of *H. pylori* was assessed at 4 weeks after inoculation. Eleven of these 23 isolates showed greater than 80% of mice positive for *H. pylori*. At 9 to 10 weeks after inoculation, the number of isolates which continued to give at least 80% colonization was reduced (5 of

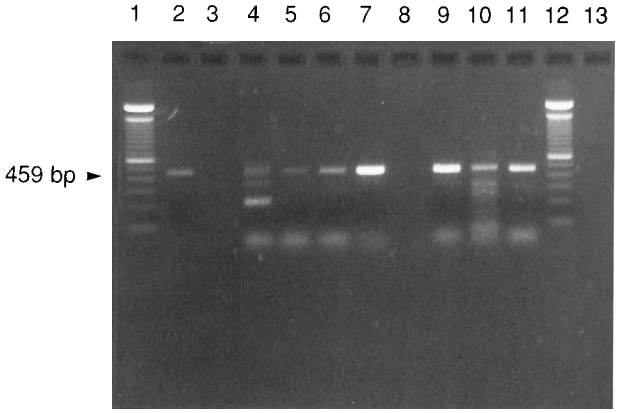

FIG. 2. PCR products from *H. pylori* (CL5173) DNA from pure culture, infected mouse stomach mucosa, and reisolated organisms. Lanes 1 and 12, DNA ladder; lane 2, DNA from 2,000 cells of pure culture; lanes 3, 8 and 13, deionized H_2O; lane 4, DNA from *H. pylori* spiked into stomach mucosal tissue; lanes 5 to 7, *H. pylori* reisolated from infected stomach mucosa at 15 min (lane 5), 1 week (lane 6), and 5 weeks (lane 7) after oral inoculation; lanes 9, 10, and 11, PCR products from stomach mucosa of mice 4 weeks after oral inoculation.

TABLE 1. PCR detection of colonization in VAF mouse strains following oral inoculation with *H. pylori* CL3498[a]

Mouse strain	% of animals positive by PCR
Outbred	
Swiss-Webster	100
CD-1	100
CD-1 + RB6-8C5 MAb (anti-Gr1)[b]	100
Inbred	
Transgenic CD4$^-$	100
SCID	100
DBA/2	100

[a] Mice were inoculated three times within a 1-week period, and stomach mucosal samples were assayed 4 weeks after initial inoculation. Four to six mice were tested per strain.
[b] These mice received injections of 60 μg of RB6-8C5 monoclonal antibody (MAb) 24 and 1 h prior to initial challenge.

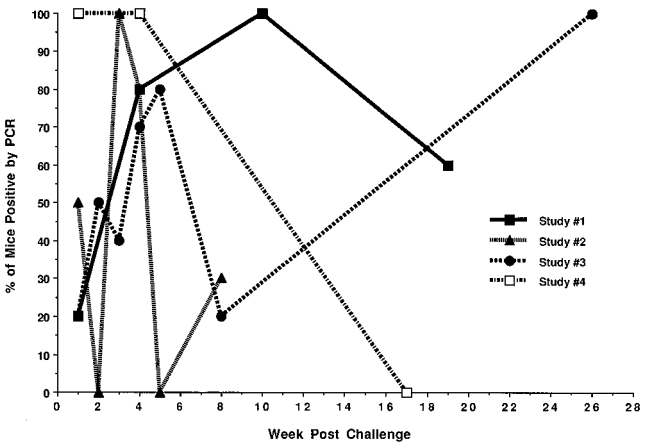

FIG. 3. Variability of colonization by *H. pylori* (CL3501) in conventional VAF Swiss-Webster mice.

15 isolates). Only 2 of the 12 isolates tested showed colonization in at least 80% of the mice at 16 weeks postchallenge. One isolate, CL3501, was assessed at 6 months and 1 year after oral inoculation, with 100% of the mice positive by PCR at 6 months and 60% positive at 1 year.

Some test-to-test variation in the percentage of mice positive by PCR following oral challenge with *H. pylori* isolates has been noted (Fig. 3). With isolate CL3501, we observed more variability between studies at the early (<2 weeks) and later (>8 weeks) time points. A more consistent, high percentage of mice were colonized with *H. pylori* at 4 weeks (70 to 100% of mice testing positive by PCR). In one study, 100% of mice were positive by PCR at 6 months and 60% of the mice tested were still positive by PCR analysis at 1 year after inoculation. To test whether some of the variability observed was related to levels of colonization near our limit of detection, we amplified multiple aliquots from stomach DNA samples in groups which were not 100% PCR positive. Testing multiple DNA aliquots from samples did not result in substantially increased percentages of PCR-positive mice within a study group. With this approach, one study group increased from 50 to 60% and a second group increased from 80 to 90% of mice positive by PCR (data not shown).

Quantitation of tissue load. Stomach mucosal samples from 3 weeks, 4 weeks, and 6 months after challenge with *H. pylori* CL3501 were used to quantitate tissue load with an adapted PCR methodology. DNA extracted from adjusted cell numbers of isolate CL3501 was used to set up a standard curve (Fig. 4). It was found, consistently, that 1 log more control DNA in a reaction mixture resulted in an equal-sized product band with *H. pylori* DNA in the standard curve reactions. When DNA from 0.5 $\log_{10}$ dilution of *H. pylori* CL3501 cell numbers was run in the same PCR with a known amount of control DNA, 10^3 molecules of control DNA resulted in a product equivalent in visualization to 10^2 *H. pylori* cells (Fig. 4, lane 5). The ratio of 10:1 for control DNA to *H. pylori* cells was observed to produce equal band products in all reactions within the linear range of the standard curve; i.e., 10^2 molecules of control DNA resulted in a product equivalent in visualization to DNA representing 10 *H. pylori* cells (other standard curve reactions not shown). The linear portion of the titration in standard curve reactions was used to determine control DNA levels to be utilized for tissue quantitation. A standard curve checkerboard titration was run simultaneously with the infected tissue analysis for accurate quantitation based on the linear range for PCRs.

Three individual stomach mucosa samples collected 3 weeks postchallenge and pooled mucosa samples (pools of six PCR-positive samples each) from 4 weeks and 6 months postinoculation were assayed against a titration of control DNA. For each stomach mucosa sample, the reaction mixture that gave equivalent PCR products with control DNA was selected and the tissue load of *H. pylori* was determined within a half-log range on the basis of comparison with the standard curve. All

TABLE 2. Screening of *H. pylori* isolates in VAF Swiss-Webster mice[a]

Time postinoculation (wk)	No. of isolates tested	No. of isolates colonized[b]
1	11	8
4	23	11
9–10	15	5
>16	12	2

[a] Mice were inoculated two to three times within a 1-week period, and stomach mucosal samples from 5 to 10 mice were assayed by PCR after initial inoculation.
[b] Colonized = >80% of mice positive for *H. pylori* by PCR analysis.

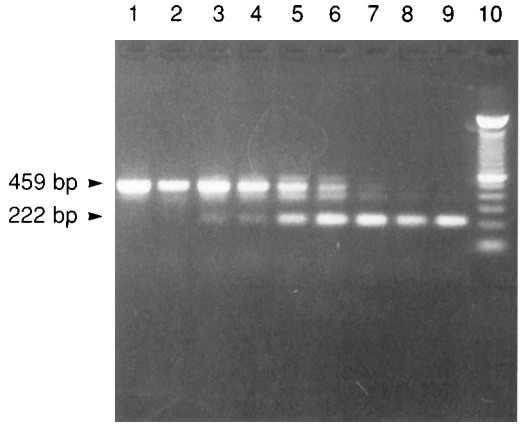

FIG. 4. PCR quantitation reaction standard curve with *H. pylori* (CL3501). Lanes 1 to 9 contain PCR products from DNA half-$\log_{10}$ dilutions of *H. pylori* (CL3501) from 10^4 (lane 1) to 1 (lane 9) cell, and each reaction (lane) also contained 10^3 molecules of control DNA. Lane 10, DNA ladder.

infected stomach mucosal samples were found to contain approximately 10^3 to 10^4 *H. pylori* cells per infected stomach.

Confirmation of mouse infectivity by reisolation. GF mice were inoculated with *H. pylori* CL5173. At 15 min, 1 week, and 5 weeks after inoculation, stomach mucosa samples were collected and plated onto HP medium to reisolate the organism. *H. pylori* was reisolated from the stomach mucosa at all three time points tested and confirmed by Gram stain, urease testing, and oxidase testing. Reisolated bacteria were processed for DNA, amplified with the specific PCR primers, and further confirmed to be *H. pylori* (Fig. 2).

DISCUSSION

We have described a new method for detection of infectivity by *H. pylori* in mice following oral inoculation. The PCR methodology is increasingly being utilized for detection of *H. pylori* in clinical studies (42, 43) due to its sensitivity and specificity. The primers we have designed were based on the 16S rRNA gene, as are the other primers recently described, due to the highly conserved nature of the gene within species. By selecting primers which had numerous base pair mismatches at the 3′ end of our upstream primer, compared with the sequences of closely related *Helicobacter* and *Campylobacter* species, we have obtained primers that exhibit excellent specificity and no observable cross-reactivity. The PCR assay described is extremely sensitive, able to detect a single copy of the 16S rRNA gene in DNA extracted from pure bacterial culture. There was only a 10-fold reduction in the sensitivity of the assay in the presence of DNA extracted from stomach mucosal tissue. Therefore, the PCR assay could detect as few as 2 organisms per reaction, corresponding to 100 organisms per whole stomach mucosa sample, when assayed in the presence of stomach mucosal DNA.

This is an extremely sensitive method of detection that affords great advantages over other methods presently used in animal models. The urease reaction is much less sensitive than PCR and requires approximately 10^6 organisms to obtain an observable reaction in either a macro- or a microassay compared with 0.2 organism detected by PCR. While urease testing is still feasible in a GF mouse model with high levels of colonization, this method of detection is more difficult to utilize with conventional mice, which have lower levels of *H. pylori* colonization and in which the presence of other urease-producing microflora could lead to improper diagnosis (44). Urease testing is always accompanied by a histopathological analysis of tissue and/or culture of organism. Once again, these methodologies are easier to accomplish utilizing GF animals in which there have been noted histopathological changes (21, 22) and in which there would be no competing organisms to be concerned with in reisolation techniques. These remain complex techniques which are difficult to assess in the case of low levels of infectivity. Culture of fastidious organisms such as *Helicobacter* spp. from tissue can be difficult in any instance and is not always 100% successful. The exact cause of histological changes in tissue is normally inferred, and changes resulting from a secondary cause cannot always be easily ruled out. Additionally, PCR does not require proliferating cells for detection, whereas most other methods of detection are reliant on active metabolism or cell growth. Therefore, use of PCR methodology in animal models, and especially in conventional animals harboring other microflora, offers distinct advantages over methodologies previously described.

We were able to achieve long-term colonization in VAF, conventional mice as opposed to the transient colonization previously reported in conventional, euthymic mice (22) and over a more extended period than reported recently in specific-pathogen-free mice (28). With 5 of 15 isolates, colonization was detected in greater than 80% of mice sampled at 9 to 10 weeks after oral inoculation. Colonization levels of 80% were achieved more than 16 weeks postchallenge with 2 of 12 isolates tested. One *H. pylori* isolate colonized 100% of mice at 6 months and 60% at 1 year after inoculation. Quantitative PCR showed the level of infectivity at 6 months to be on the order of 10^4 organisms per stomach. The sensitivity of PCR allowed detection of *H. pylori* in mice at these later time points, while other methodologies could not. Screening of different isolates in conventional mice indicated that choice of isolate used for model development may be critical for achieving long-term colonization.

We did observe some experiment-to-experiment variability in colonization, even with isolates which colonized at high levels (>80% of mice positive by PCR) in some long-term studies. We cannot account for this variability, which was observed at different time points in various studies. It has been reported that fresh, unfrozen clinical isolates of *H. pylori* were capable of colonizing specific-pathogen-free and conventional mice whereas a laboratory strain was unable to establish infection (28). It may be that freezing our clinical isolates contributed to the observed variability. Cultures of *H. pylori* for individual studies were prepared from frozen stock aliquots of a selected clinical isolate. The cultures for challenge had to be scaled up from frozen aliquots but were always used at the second to fourth passage from the frozen stock. The Swiss mice were obtained from the same breeder and had the same initial microflora. The viability of challenge cultures was tested by plating on culture media, and while there was some variability in the inoculum size, there was no correlation between challenge inoculum and variation in colonization potential. The potential for colonization at the limits of PCR detection of 100 organisms per stomach sample was considered. Multiple-aliquot testing in PCRs to increase the potential of *H. pylori* DNA being present for amplification did not result in a substantially enhanced percentage of samples testing positive by PCR. Quantitation of stomach mucosa samples at multiple time points consistently showed 10^3 to 10^4 organisms per stomach, in agreement with levels of colonization detected by culture in GF mice (22). The possible role of *Lactobacillus* spp. in affecting colonization of *H. pylori* in conventional euthymic mice has been proposed by Karita and colleagues (22). The effect of the presence of *Lactobacillus acidophilus* or its metabolites on inhibition of bacterial growth has been noted for *H. pylori* in vitro (3) and for other models of mucosal pathogens (4, 32, 34, 36). This could account for some of the variability observed, as *Lactobacillus* sp. is a constituent of the defined flora of the mice utilized in these studies. The potential of some *H. pylori* isolates to better compete with *Lactobacillus* spp. or evade any metabolites or immune mechanisms resulting from the presence of *Lactobacillus* spp. may be responsible for the long-term colonization success of a small percentage of isolates.

To confirm that the PCR was detecting infection of viable organisms, GF mice were inoculated with *H. pylori* and the organism was reisolated following inoculation. *H. pylori* was successfully reisolated from mice at 15 min and 1 and 5 weeks postchallenge. The reisolates were identified by urease and oxidase testing along with Gram stain and then confirmed by PCR amplification. The amplified products from the reisolates were identical to those observed from infected stomach mucosal samples. We tried to perform the same reisolation studies in conventional mice but were unsuccessful each time due to the presence of other microflora which competitively prevented or masked growth of *H. pylori* in culture. Also, we

encountered urease-producing *Proteus* sp. in our mice after they were maintained in our animal facility, which rendered use of urease testing for diagnosis meaningless in these studies. From our experience, use of standard techniques in a conventional mouse model appears to be highly unreliable for detection of colonization by *H. pylori*. However, we were able to detect *H. pylori*-specific antibodies by enzyme-linked immunosorbent assay (data not shown) in the sera of conventional mice 2 and 3 weeks after inoculation, further supporting the evidence that the mice were colonized.

PCR is both specific and sensitive compared with previously used techniques for the detection of tissue colonization. This methodology has allowed detection of colonization by *H. pylori* following oral inoculation in conventional, euthymic mice over extended periods of time up to 1 year following inoculation. PCR will allow development of *Helicobacter* models which do not necessitate use of GF animals for consistent and reliable determination of colonization. Further refinement of a conventional mouse model of *H. pylori* can now be pursued for use in studies aimed at developing both new therapeutics and vaccines, as well as for the basic study of the disease process. Additionally, PCR as a tool in animal models will lead to more accurate assessment of the outcome of chemotherapeutic or vaccine regimens with regard to eradication or reduction of organism.

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