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Evaluation of a Commercial Colorimetric Fecal Dipstick Assay for the Detection of *Helicobacter hepaticus* Infections in Laboratory Mice

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Mice used in biomedical research typically are tested for the presence of *Helicobacter* spp., including *Helicobacter hepaticus*. Here we evaluated the ability of a commercially available colorimetric *Helicobacter* dipstick assay to detect *H. hepaticus* in experimentally and naturally infected mice, with use of a *Helicobacter* PCR assay as the 'gold standard' test. None of the fecal samples from experimentally infected A/JCr mice (n = 12) tested positive for *Helicobacter* by the colorimetric dipstick test. In naturally infected A/JCr and C57BL/6 mice, 11% (1 of 9) and 30% (3 of 10) of fecal samples, respectively, tested positive for *Helicobacter* by the colorimetric dipstick assay. In these 3 groups of *H. hepaticus*-infected mice, statistically fewer mice tested positive by the colorimetric dipstick test than by PCR. The colorimetric *Helicobacter* dipstick assay had an overall diagnostic sensitivity of 13%, diagnostic specificity of 94%, and analytical sensitivity of 10^8 *H. hepaticus* cfu/mL. As currently formulated, the colorimetric dipstick assay had high specificity but lacked sensitivity for detecting *H. hepaticus* infections in 2 strains of mice commonly used in research, thereby limiting its utility as a diagnostic screening test for *H. hepaticus* infections in research mice.

Recent surveys indicate that members of the genus *Helicobacter* are among the most often detected bacterial pathogens in the gastrointestinal tracts of laboratory mice.[4,9,13] At least 8 *Helicobacter* spp. naturally infect mice used in biomedical research.[8,16,17] Of these, *Helicobacter hepaticus* has been reported to be the most prevalent pathogenic species of *Helicobacter* detected in laboratory mouse colonies.[4,8,9] Consequences of infection with *H. hepaticus* range from subclinical disease to overt morbidity in susceptible mouse strains. In addition, hepatic and colonic neoplasia, hepatitis, and typhlocolitis have been associated with *H. hepaticus* infection in multiple strains of mice.[4,8,14,16,17] Two such strains, A/JCr and C57BL/6, differ in their response to *H. hepaticus* infections, with A/JCr mice susceptible and C57BL/6 mice resistant to hepatic and large intestinal pathology.[15]

Identification of *Helicobacter* positive mice is an integral component of many health monitoring programs. Accurate detection of *Helicobacter* spp. is crucial to maintain *Helicobacter*-free mouse colonies and minimize the potential for these pathogens to confound research studies, especially those involving enterohepatic disease models.[7] Diagnostic approaches to detect *Helicobacter* infections in mice include microbiologic culture of intestinal contents or feces, serologic assays for the detection of *Helicobacter*-specific antibodies in serum, histopathologic evaluation of liver or large bowel, and PCR testing of feces or tissues. Currently, evaluation of feces by PCR is the 'gold standard' diagnostic assay for detecting enteric *Helicobacter* infections in mice because these assays provide the highest sensitivity, specificity, and convenience for identifying *Helicobacter* infections.[1,2,12,17] Recently, an assay was made commercially available that is a one-step rapid screening test for qualitative detection of *Helicobacter* infections. The colorimetric assay uses an enzymatic reaction, and subsequent color change, to identify *Helicobacter* antigen in infected mouse feces. Although results can be obtained rapidly, data regarding the diagnostic performance of this assay are not available. The purpose of the present studies was to evaluate the diagnostic performance of the colorimetric assay by using a fecal PCR assay as the gold standard test for detecting *Helicobacter* infections in research mice. As such, the sensitivity and specificity of the colorimetric dipstick test was evaluated in A/JCr mice experimentally infected and C57BL/6 and A/JCr mice naturally infected with *H. hepaticus*, a highly prevalent, pathogenic enteric *Helicobacter*.

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Materials and Methods

Animal care. All mouse manipulations were performed in a class II biological safety cabinet. All experimental mice were group-housed at 2 to 4 mice per cage in static filter-top cages and provided autoclaved, acidified water and standard irradiated rodent chow ad libitum. All animal procedures were done in accordance with *Guide for the Care and Use of Laboratory Animals*[3] and the animal care policies of the University of Missouri–Columbia Institutional Animal Care and Use Committee.

***Helicobacter* colorimetric dipstick assay.** The *Helicobacter* colorimetric dipstick assay (Helico-Stix, Lab Etc., Clayton, DE), a *Helicobacter* antigen test that utilizes a capture reagent on a precoated membrane, was performed according to the manufacturer's instructions. The same fecal samples were also tested by *Helicobacter* PCR after performance of the colorimetric dipstick assay. Fresh (no more than 24 h old) fecal pellets were collected from control and experimental mice as directed. Mice were placed individually in autoclaved filter-top cages without bedding, and 8 to 10 voided fecal pellets per mouse were collected and placed in a marked 2.0-mL tube. Deionized sterile water (1 mL) was added to each sample. All samples were vortexed continuously for 1 min to dissolve the contents. The resultant slurry was cloudy with some particulate material

visible. Subsequent to mixing, the sample was left to stand at room temperature for 2 to 5 min. The indicator reagent pad was immersed into the fecal mixture, the test strip was removed, and the reagent pad evaluated for a color change at the 20- to 30-s time point. The product literature indicated that a color change of blue to greenish-blue was indicative of a positive result; no color change, in which the strip remained a yellow color, was indicative of a negative result.

The analytical sensitivity of the colorimetric dipstick assay was determined by testing serial dilutions of broth cultured *H. hepaticus* enumerated by quantitative PCR. Serial 1:10 dilutions of *Helicobacter hepaticus* were made by using sterile distilled water as the diluent. The colorimetric dipstick assay was performed and interpreted as described.

Isolation of DNA and *Helicobacter* PCR analysis. After the *Helicobacter* dipstick test was performed, the fecal slurries then were processed for *Helicobacter* PCR. Slurries were homogenized further with a stainless steel ball (Tissuelyser, Qiagen,Valencia, CA) for 30 seconds at 30 Hz. Next, the samples were centrifuged for 5 min at 1600 × *g*, and DNA was isolated from 200 µL of the resulting supernatant by using a commercial kit (DNeasy, Qiagen) according to manufacturer's protocol. In addition, isolation of DNA from broth-cultured *H. hepaticus* was performed as described, with the exception that DNA was isolated directly from 200 µL bacterial culture. *Helicobacter* genus-specific primers (276F 5′ CTA TGA CGG GTA TCC GGC 3′ and 676R 5′ ATT CCA CCT ACC TCT CCC A 3′) designed to complement regions of the 16S rRNA gene conserved among members of the *Helicobacter* genus were used in a *Helicobacter* PCR assay as previously described.[1,11] Each 50-µL PCR reaction contained 1 µM each primer, 200 µM each dNTP (dATP, dCTP, dGTP, dTTP), PCR buffer (50 mM Tris-HCl, 10 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$ [pH 8.3]), 1.25 U FastStart *Taq* DNA polymerase (Roche Diagnostics, Mannheim, Germany), 5 µL extracted DNA from the fecal slurry, and nuclease-free water. Amplification was performed in a thermal cycler with the following conditions: 95 °C for 4 min, followed by 45 cycles of denaturation at 94 °C for 30 s, primer annealing at 62 °C for 30 s, and extension at 72 °C for 30 s. A 15-µL volume of each PCR product underwent electrophoresis on a 3% agarose precast gel with ethidium bromide (BioRad Laboratories, Hercules, CA) and was visualized under UV light.

The analytical sensitivity of the *Helicobacter* PCR assay was determined by testing serial dilutions of DNA extracted from broth-cultured *H. hepaticus* enumerated by quantitative PCR. Serial 1:10 dilutions of extracted DNA were made by using sterile distilled water as the diluent. The *Helicobacter* PCR assay was performed and interpreted as described.

Quantitative PCR analysis. The numbers of *H. hepaticus* organisms present in *H. hepaticus* cultures and fecal slurries from experimentally infected mice were determined by using quantitative PCR that targeted the *H. hepaticus cdtB* gene as previously described.[5] DNA was extracted from bacterial cultures and fecal slurries as described.

***H. hepaticus* cultivation.** A *H. hepaticus* isolate, strain MU94, was obtained from an endemically infected mouse colony. The isolate was identified as *H. hepaticus* by morphology, biochemical characteristics, and sequence analysis of the 16S rRNA gene.[1] *H. hepaticus* cultures were grown in 250 mL *Brucella* broth and incubated for 48 h at 37 °C in a microaerobic environment with 90% N_2 /5% H_2 /5% CO_2.[5]Fresh cultures were used for mouse inoculations. Bacteria used for other experiments were frozen at –80 °C until use.

Animals and specimen collection. Female A/JCr mice (age, 4 wk) were obtained for experimental inoculation and natural infection studies; in addition, 4-wk-old, female C57BL/6 mice were obtained for natural infection studies. All mice were obtained from Frederick Cancer Research and Development Center (Frederick, MD) and were free of ectoparasites, endoparasites, *Mycoplasma pulmonis*, *Helicobacter* spp., known enteric and respiratory bacterial pathogens, and antibodies to mouse hepatitis virus, Sendai virus, pneumonia virus of mice, reovirus 3, Theiler murine encephalomyelitis virus, ectromelia virus, polyoma virus, lymphocytic choriomeningitis virus, mouse adenovirus, minute virus of mice, mouse parvovirus, mouse rotavirus, mouse cytomegalovirus, mouse thymic virus, *Encephalitozoon cuniculi*, and *Clostridium piliforme*.

For experimental inoculation studies, A/JCr mice (n = 12) were inoculated by oral gavage with 10^8 *H. hepaticus* organisms in 0.5 mL *Brucella* broth; A/JCr control mice (n = 12) were inoculated by oral gavage with 0.5 mL *Brucella* broth. At 2 mo after inoculation, feces were collected from individual mice according to the instructions from the dipstick assay's manufacturer. Briefly, mice were transferred to individual autoclaved filter-top cages without bedding, and 8 to 10 of the 'freshest' fecal pellets were collected from each cage the next morning by using clean gloved hands or sterile forceps. To model natural infection, naïve A/JCr (n = 9) and C57BL/6 (n = 10) mice were exposed for 3 wk to dirty bedding from mice experimentally infected with *H. hepaticus*. In addition, a single mouse experimentally infected with *H. hepaticus* was placed in each cage to permit direct contact exposure. Uninfected A/JCr (n = 3) and C57BL/6 (n = 3) mice similarly obtained from Frederick Cancer Research and Development Center were used as controls in natural infection studies and evaluated at the cage level. The *H. hepaticus* infection status of all experimental and control mice in the experimental and natural infection experiments was determined by using a generic *Helicobacter* PCR assay,[1] and each mouse was further confirmed to be monoinfected with *H. hepaticus* by a multiplex PCR assay.[10]

Statistical analysis and assay performance. The Fisher exact test (SigmaPlot 11.0, Systat Software, San Jose, CA) was used to test for differences in the number of samples testing positive by the colorimetric dipstick test and the *Helicobacter* PCR assay. P values less than 0.01 were considered statistically significant.

The sensitivity and specificity of the colorimetric dipstick assay in detecting *H. hepaticus* in feces was evaluated by using the *Helicobacter* PCR assay as the gold standard test and the following formulas:

$$\text{sensitivity} = [\text{true positives}/(\text{true positives} + \text{false negatives})] \times 100\%$$

$$\text{specificity} = [\text{true negatives}/(\text{true negatives} + \text{false positives})] \times 100\%$$

Results

Detection of *H. hepaticus* in feces from experimentally and naturally infected mice. The diagnostic performance of the *Helicobacter* dipstick test was evaluated for its ability to detect *H. hepaticus* in the feces of mice, with a fecal *Helicobacter* PCR assay being used as the gold standard test. Experimentally and naturally infected A/JCr mice were evaluated to account for potential differences in *H. hepaticus* fecal shedding that could result from the method of infection. In addition, naturally infected A/JCr mice and C57BL/6 mice were evaluated to account for potential differences in *H. hepaticus* fecal shedding that could result from differences between A/JCr mice and C57BL/6 mice,

which represent mouse strains that are susceptible or resistant to *H. hepaticus*-induced disease, respectively.

At 2 mo after inoculation, the feces of all inoculated mice (n = 12) tested positive for *H. hepaticus*, and the feces of all sham-inoculated control mice (n = 12) tested negative for *H. hepaticus* by the fecal *Helicobacter* PCR assay, thereby documenting the *H. hepaticus* infection status of these mice. The same feces from all *H. hepaticus*-infected mice (n = 12) and all uninfected control mice (n = 12) tested negative for *Helicobacter* with the colorimetric dipstick assay (Figure 1; data from uninfected control mice not shown). The number of mice testing positive by the colorimetric dipstick differed significantly ($P < 0.01$) from the number found with the *Helicobacter* PCR assay. On the basis of these findings, the *Helicobacter* dipstick test had a sensitivity of 0% and a specificity of 100% for detecting *H. hepaticus* in the feces of A/JCr mice experimentally infected with *H. hepaticus*.

The *Helicobacter* dipstick assay was further evaluated for its capacity to detect *H. hepaticus* in the feces of naturally infected mice. Groups of A/JCr mice (n = 9) or C57BL/6 mice (n = 10) each were cohoused with a single *H. hepaticus*-infected mouse and exposed to dirty bedding from *H. hepaticus*-infected mice for 3 wk to allow mice to become infected with *H. hepaticus*. Infection with *H. hepaticus* was confirmed in that all A/JCr mice (n = 9) and C57BL/6 mice (n = 10) tested positive for *Helicobacter* by the fecal PCR assay. In testing fecal samples from these mice with the *Helicobacter* dipstick assay, 11% (1 of 9) of the feces from naturally infected A/JCr mice and 30% (3 of 10) of the feces from naturally infected C57BL/6 mice tested positive by the *Helicobacter* dipstick assay (Figure 1). The number of mice testing positive by the colorimetric dipstick differed significantly ($P <$ 0.01) from the number found with the *Helicobacter* PCR assay in both mouse strains evaluated. Uninfected control mice from both mouse strains (n = 3 samples per strain) documented to be negative for *Helicobacter* by PCR were also tested with the colorimetric fecal dipstick. All 3 fecal samples from C57BL/6 mice and 2 of 3 fecal samples from A/JCr mice tested negative, whereas one fecal sample from an uninfected control A/JCr mouse tested positive by the dipstick (data not shown).

Combining all colorimetric *Helicobacter* dipstick testing data from experimentally and naturally infected mice, the colorimetric *Helicobacter* dipstick test had an overall diagnostic sensitivity of 13%, with 4 of 31 known *H. hepaticus*-positive fecal samples testing positive by the dipstick, and an overall diagnostic specificity of 94%, with 17 of 18 known *Helicobacter*-negative fecal samples testing negative by the dipstick.

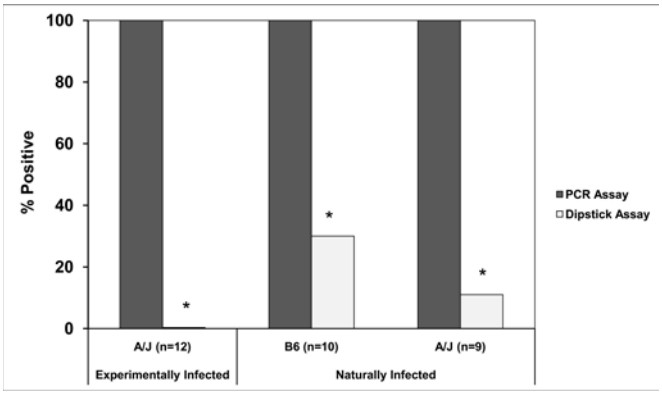

Figure 1. Percentages of fecal samples from A/JCr mice (A/J; n = 12) experimentally inoculated with *H. hepaticus* and A/JCr mice (n = 9) and C57BL/6 mice (B6; n = 10) naturally infected with *H. hepaticus* that tested positive for *Helicobacter* spp. by a colorimetric dipstick assay or a *Helicobacter* PCR assay. The same fecal sample from individual mice was tested by using both assays. The number of samples testing positive or negative was used for statistical analysis. *, $P < 0.01$ compared with mice of the same strain and method of infection.

Detection of cultured *H. hepaticus*. The detection limits of the colorimetric *Helicobacter* test and the *Helicobacter* PCR assay were determined by using *H. hepaticus* propagated in vitro. The number of *H. hepaticus* organisms per milliliter of culture was established by using qualitative PCR on serial 10-fold dilutions of *H. hepaticus* ranging from 10^0 to 10^9 cfu/mL. The *Helicobacter* dipstick test produced a positive result for bacterial suspensions with 10^9 and 10^8 *H. hepaticus* cfu/mL; suspensions containing 10^7 *H. hepaticus* cfu/mL or fewer tested negative (data not shown). Therefore, the minimal detection limit of cultured *H. hepaticus* with the *Helicobacter* dipstick test was determined to be 10^8 organisms per milliliter. The *Helicobacter* PCR performed on the same samples yielded a positive result on all dilutions of *H. hepaticus* at concentrations at or above 10^3 *H. hepaticus* cfu/mL. This result equates to a detection limit of 10 copies of *H. hepaticus* per PCR reaction, given that at the lowest bacterial dilution that tested positive (10^3 *H. hepaticus* per milliliter), the 200 µL bacterial culture processed for DNA contained 200 bacteria, the DNA extract (100 µL) yielded a DNA solution containing 200 bacterial genomes per 100 µL, and 5 µL of this DNA extract was used in each PCR reaction. Given these data, the *Helicobacter* dipstick test required a concentration of bacteria 100,000 times that of the *Helicobacter* PCR assay to yield a positive result.

Quantitation of *H. hepaticus* in fecal slurries of experimentally infected mice. A quantitative *Helicobacter* PCR assay was performed to determine the concentration of *H. hepaticus* in the fecal samples of the experimentally infected mice used to evaluate the *Helicobacter* dipstick test. The number of genome copies of *H. hepaticus* in the DNA extracted from the 12 fecal samples from the A/J mice experimentally infected with *H. hepaticus* ranged from 6.6×10^3 to 1.9×10^5 copies per PCR reaction. Assuming 100% recovery of *H. hepaticus* genome during DNA extraction from feces, each sample containing 8 to 10 fecal pellets had a minimum of 1.7×10^6 to 4.8×10^7 *H. hepaticus* organisms per milliliter of fecal slurry evaluated with the *Helicobacter* dipstick test.

Discussion

In the studies reported herein, we evaluated the sensitivity and specificity of a commercial colorimetric dipstick test designed to detect *Helicobacter* in mouse feces. Although the *Helicobacter* dipstick test had adequate test specificity and produced only one false-positive test result, test sensitivity was low in that the assay failed to detect *H. hepaticus* in the feces of any experimentally infected A/JCr mice and detected *H. hepaticus* in only 21% of naturally infected mice. The reason for the low sensitivity of the colorimetric dipstick test is likely due to the high bacterial concentration needed for the assay to yield a positive result. When the *Helicobacter* dipstick test was performed on dilutions of broth-cultured *H. hepaticus*, the lowest concentration of bacteria detected by the assay was 1×10^8 *cfu/mL*. This bacterial concentration is 2 to 59 times higher than was obtained from the feces of A/JCr mice experimentally infected with *H. hepaticus* when feces were prepared according to the dipstick test kit recommendations of combining 8 to 10 fecal pellets from a single *H. hepaticus*-infected mouse. Therefore, it is not surprising that the *Helicobacter* dipstick test did not detect *H. hepaticus* in the feces of the experimentally infected A/JCr mice. Conversely, the *Helicobacter* PCR assay has a detection limit of 100 *H. hepaticus* genome copies per PCR reaction when testing DNA extracted from feces.[1] This limit is equivalent to 1×10^4 organisms per

milliliter of fecal slurry, given how fecal samples from infected mice were prepared in this study. The concentration of *H. hepaticus* in 1 mL of the fecal slurries from the experimentally infected A/JCr evaluated was 170 to 4800 times above the detection limit of the *Helicobacter* PCR assay.

Other differences between the 2 test formats include: sample requirements, technical time, reagents and equipment, and testing cost. To perform the *Helicobacter* dipstick test, the product testing guidelines require 8 to 10 fresh (less than 24 h old) fecal pellets from a single mouse. In contrast, the *Helicobacter* PCR assay can be performed on a single mouse pellet collected immediately or within 5 d of a bedding change, and feces from multiple mice can be pooled and tested as a single sample.[1] Pooling of feces from different mice is not recommended by the distributer of the dipstick test. The sample collection requirements of the PCR assay are an advantage, because they facilitate more convenient collection of fecal samples from large colonies of mice. An advantage of the *Helicobacter* dipstick test is that it requires considerably less technician time and expertise, taking only about 5 min to complete whereas PCR takes approximately 8 h to complete. However, the potential advantages of the *Helicobacter* colorimetric assay to provide a rapid, easy, and cageside test for *Helicobacter* infections are negated by its low sensitivity in detecting *H. hepaticus*-infected mice.

Several *Helicobacter* species have been identified in laboratory mice. Of these, *H. hepaticus* is considered to be among the most pathogenic and prevalent in mice.[4,8,9,14,16,17] The current studies compared the rapid, one-step *Helicobacter* dipstick test to a *Helicobacter* PCR assay for detection of *H. hepaticus*-contaminated feces from experimentally and naturally infected mice; other less-prevalent rodent *Helicobacter* spp. were not evaluated. However, the low sensitivity of the colorimetric dipstick assay to detect *H. hepaticus* infections in mice limits the utility of this test as a diagnostic assay to screen mice used in biomedical research.

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