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# Diagnosis of *Helicobacter pylori* Infection in a Colony of Rhesus Monkeys (*Macaca mulatta*)

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**Twenty-three young adult rhesus monkeys from China were evaluated for the presence of *Helicobacter pylori*. Gastric body and antral biopsy samples were tested for *H. pylori* by PCR analysis, culture, rapid urease testing, and histologic evaluation. Serologic testing to detect *H. pylori* immunoglobulin G (IgG) antibodies was performed by using a commercially available human-based enzyme-linked immunosorbent assay (ELISA) test and an ELISA test which utilized homologous *H. pylori* antigens and an anti-rhesus IgG conjugate. PCR analysis with *H. pylori*-specific 26-kDa protein primers detected *H. pylori* in 21 of the 23 rhesus monkeys (91%). Culture testing identified the organism in 12 of the 23 animals (52%). Rapid urease tests were positive for all animals. *H. pylori* was diagnosed by histological examination in 11 of 23 monkeys (48%). Of the 21 monkeys positive for *H. pylori* by PCR, only 3 (14%) had positive results by the commercial ELISA test, yielding a sensitivity of 14%, a specificity of 100%, and an accuracy of 22%. However, 19 of the 21 PCR-positive animals (90%) had positive results by the ELISA test with homologous rhesus *H. pylori* antigen and anti-monkey conjugate, with predicted index values greater than or equal to 0.7 considered positive and values between 0.5 and 0.7 considered equivocal. This test had a sensitivity of 90%, a specificity of 100%, and an accuracy of 91%. Therefore, the ELISA test with rhesus monkey origin components was more accurate for detecting infected animals than the human-based ELISA.**

*Helicobacter pylori* is now established as a human pathogen. It is the causative agent of chronic active gastritis and duodenal ulcer formation and recurrence and a probable cofactor in the development of gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma in humans (3, 8–10, 14, 15, 20, 27). Because of the importance of this bacterium in gastric disease in humans, there has been an increasing need for accurate, efficient methods of detection of *H. pylori.*

Early methods of diagnosing *H. pylori* in humans, all of which utilized gastric biopsy samples, included evaluation of Gram-stained impression smears, rapid urease testing, histological examination, and culture. PCR methodology has been used recently to test gastric biopsy samples for the presence of *H. pylori* (11). PCR testing is valuable because of its superior sensitivity compared to other methods.

Serological assays are also being used to diagnose *H. pylori* infection. Infected humans consistently develop a systemic humoral immunoglobulin G (IgG) immune response to the organism (21, 26). Enzyme-linked immunosorbent assay (ELISA) testing for IgG antibodies is the serologic method of choice for the diagnosis of *H. pylori*. ELISA testing has been described as a highly accurate and reliable method for the diagnosis of *H. pylori* in humans (25).

The rhesus monkey (*Macaca mulatta*) has been proposed as a model for the study of *H. pylori* because of its availability, its consistent development of gastritis in response to *H. pylori*, and its high rate of natural infection with the organism (1, 4, 6, 7, 18, 19, 23).

The goal of this study was to evaluate the following methods for detection of *H. pylori* in the rhesus monkey: PCR, culture, rapid urease testing, and histologic examination. We also compared the ability of a commercially available ELISA kit that contained human-based reagents with that of an ELISA test utilizing anti-rhesus monkey conjugates and *H. pylori* isolated from the infected monkeys as antigen substrate for detecting *H. pylori* infection.

## MATERIALS AND METHODS

**Animals.** A colony of 23 young adult rhesus monkeys, which originated in China, was surveyed for *H. pylori*. After arrival, the animals were individually housed indoors, separate from other groups of monkeys, in stainless steel cages at an American Association for Accreditation of Laboratory Animal Care-accredited facility. Water was provided ad libitum through an automatic watering system and high-protein monkey diet number 5045 (PMI Feeds, St. Louis, Mo.) was fed twice daily. The animals ranged from approximately 1 to 3 years of age and weighed from 2.0 to 3.5 kg. None of the monkeys had clinical signs referable to gastrointestinal disease during the course of the study. The study was approved by the Merck Research Laboratories Institutional Animal Care and Use Committee.

**Gastroscopy.** The monkeys were sedated with an intramuscular injection of a ketamine-xylazine mixture (ketamine, 11 mg/kg of body weight; xylazine, 0.6 mg/kg) and anesthetized with isoflurane. The animals were endoscoped, and six to eight mucosal biopsy samples were taken from the body and antral portions of the stomach.

**PCR.** One biopsy specimen from the body and one from the antral regions of each monkey's stomach were placed in a solution containing 70% brucella broth, 20% fetal bovine serum, and 10% glycerol. These samples were frozen at −70°C prior to PCR analysis.

**DNA extraction.** DNA was extracted from frozen biopsy samples. Approximately 15 mg of tissue was homogenized to uniformity with a plastic, microcentrifuge-adapted pestle. Tissue was then processed with the Rapid Prep genomic DNA kit as outlined by the manufacturer (Pharmacia Biotech, Piscataway, N.J.).

**PCR amplification of *H. pylori*.** The selection of primers for PCR amplification was based on previously published data detailing the specificity and sensitivity of these primers (11). The primer pair designated P3 and P4 specifically amplifies a 298-bp sequence and contains the following sequences: 5′-TGGCGTGTCTA TTGACAGCGAGC-3′ and 5′-CCTGCTGGGCATACTTCACCATG-3′. These oligonucleotides are identical to residues 474 to 496 and 776 to 754 of the published sequence, respectively. The 26-kDa product amplified by these primers has not been detected in any known gastric *Helicobacter* spp. colonizing monkey stomachs other than *H. pylori* (7a).

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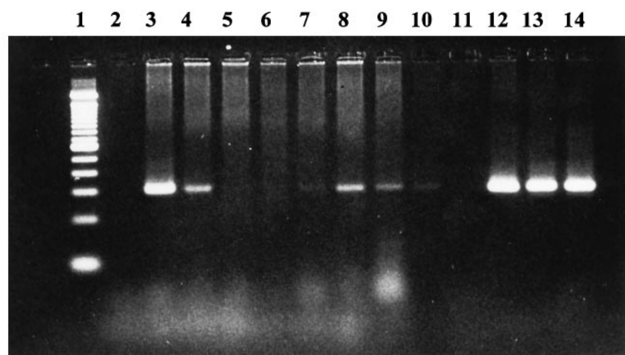


FIG. 1. PCR results for rhesus monkey biopsy samples with primers P3 and P4 that are specific for the *H. pylori* 26-kDa protein. Lanes: 1, 100-bp marker; 2, blank; 3, *H. pylori* control; 4, 881B +; 5, 877B −; 6, 876B −; 7, 869B +; 8, 811B +; 9, 857B +; 10, 875A +; 11, 858A −; 12, 866A +; 13, 855A +; 14, 816A +. In the lane designations, B refers to a body specimen, A refers to an antrum specimen, + indicates that the specimen was positive for *H. pylori*, − indicates that the specimen was negative for *H. pylori*, and the numbers are rhesus monkey designations (see Table 1).

Between 12 and 18 μl of the DNA preparation was added to 100 μl (final volume) of reaction mixture containing 1× *Taq* polymerase buffer (supplied by the manufacturer but supplemented with 1 M $MgCl_2$ to a final concentration of 2.25 mM), the two primers (each 0.5 μM), deoxynucleotides (each 200 μM), and 200 μg of bovine serum albumin per ml. Samples were heated at 94°C for 4 min, centrifuged, and cooled to 65°C. Then, 2.5 U of *Taq* polymerase (Pharmacia) and 1.0 U of polymerase enhancer (Perfect Match; Stratagene, La Jolla, Calif.) were added; this was followed by an overlay of 100 μl of mineral oil. Previously published methods were used for amplification (22).

***H. pylori* culture.** Two biopsy specimens from the body and antral regions of each stomach were collected for bacterial culture and processed as described previously (13). *H. pylori* organisms were identified as gram-negative, curved, rod-shaped bacteria, which were catalase, oxidase, and urease positive. They were negative for nitrate reduction, hippurate hydrolysis, and $H_2S$ production in triple sugar iron agar; resistant to nalidixic acid; sensitive to cephalothin; and did not grow under microaerobic conditions at 25 or 42°C.

**Urease assay.** Biopsy samples from the body and antral regions of each animal's stomach were placed into tubes containing urea agar (selective rapid urea; Remel, Lenexa, Kans.) for rapid urease testing. The tubes were incubated at room temperature, monitored hourly for 8 h, and evaluated a final time at 24 h. A pink color in the gel was considered a positive test.

**Histopathologic sections.** Two biopsy samples from the body and antral regions of each stomach were placed in 10% buffered formalin. These samples were embedded in paraffin, cut into 5-μm-thick sections, and stained with Warthin-Starry silver stain. The sections were then blind coded and evaluated for the presence and morphology of organisms. The quantity of organisms seen in each biopsy section was graded according to the following scale: 0, no organisms seen; 1, small numbers of organisms (<10 organisms/section); 2, moderate numbers of organisms (10 to 50 organisms/section), 3, large numbers of organisms (>50 organisms/section).

**ELISA (Pyloristat test).** Blood samples were collected at the time of endoscopy, and sera were separated by centrifugation at 800 × *g* for 10 min at 22°C and stored at −20°C. All serum samples were evaluated with a commercial ELISA kit containing human-based reagents (Pyloristat; Whittaker Bioproducts, Walkersville, Md.) according to the manufacturer's directions. The test used plastic wells coated with purified *H. pylori* antigen, which was prepared from a pool of sonicated *H. pylori* strains of human origin, and an alkaline phosphatase conjugate of anti-human IgG antibody.

**Modified Pyloristat test.** A portion of each serum sample was also evaluated by ELISA testing utilizing homologous *H. pylori* antigen and an anti-rhesus monkey antibody conjugate in place of the human components. Six *H. pylori* strains isolated from this colony of rhesus monkeys were grown for 5 days on blood agar plates at 37°C under microaerobic conditions. Bacteria were harvested from the plates and suspended in 10 ml of sterile phosphate-buffered saline (PBS). The cells were washed three times in PBS and were then disrupted sonically. Disruption of the bacteria was ensured by visual inspection of Gram-stained preparations. The samples were centrifuged at 4°C for 10 min at 2,670 × *g*, and the supernatant was collected. After Lowry protein analysis, the antigen was stored at −70°C in aliquots. Antigen was diluted in 0.1 M carbonate buffer, pH 9.6, to equal 1 μg of antigen per ml of buffer. Wells of flat-bottomed polystyrene microtiter plates (Dynatech, Alexandria, Va.) were coated with 100 μl of the antigen solution and left overnight at 4°C. This experiment used an alkaline phosphatase conjugate of anti-rhesus monkey IgG (Sigma, St. Louis, Mo.), diluted 1:1,000 with PBS, as the secondary antibody. All the other steps and reagents followed the protocol of the Pyloristat test kit.

The results of culture, rapid urease testing, histologic examination, and serology were compared with those obtained with PCR. A positive result by PCR was considered the reference standard for detection of *H. pylori*. The test methods were evaluated by calculation of their sensitivity, specificity, positive predictive value, negative predictive value, and accuracy (24).

## RESULTS

**PCR.** PCR analysis yielded positive results for *H. pylori* in 21 of the 23 rhesus monkeys (91%) (Fig. 1). Eight of the monkeys were positive by PCR for both the antrum and the body, 12 were positive for the antrum only, and one was positive for the body only (Table 1).

**Culture.** *H. pylori* was isolated from 12 of 23 rhesus monkeys (52%); all 12 were also positive by PCR testing. Compared to PCR, culture sensitivity was 57% and the specificity was 100% (Table 2). The positive predictive value was 100%, the negative predictive value was 18%, and the accuracy was 61%.

**Urease testing.** Rapid urease tests for either the body or antrum were positive for all animals. Compared to the results of PCR testing, the sensitivity of rapid urease testing in the rhesus monkey was 100% and the specificity was 0%. The positive predictive value was 91%, the negative predictive value could not be calculated because of the absence of negative results, and the accuracy was 91%.

TABLE 1. Results of histology, culture, and PCR testing on rhesus monkey gastric tissue

| Monkey designation | Body specimen | | Antrum specimen | |
|---|---|---|---|---|
| | Histology (W-S) results[a] | PCR results | Histology (W-S) results | PCR results |
| Culture-positive monkeys[b] | | | | |
| 811 | 3GH | + | 2HP | + |
| 812 | 2GH | + | 3HP | + |
| 814 | 3GH | + | 3HP | + |
| 816 | 3GH | − | 3HP | + |
| 855 | 3GH | − | 3HP | + |
| 866 | 3GH | − | 3HP | + |
| 872 | 3GH | + | 3HP | + |
| 874 | 3GH | − | 0 | + |
| 875 | 3GH | − | 3HP | + |
| 878 | 1GH, 1HP | − | 1HP | + |
| 880 | 3GH | − | 3HP | + |
| 884 | 3GH | − | 3HP | + |
| Culture-negative monkeys[c] | | | | |
| 807 | 1GH | + | 0 | + |
| 813 | 2GH | − | 0 | + |
| 815 | 3GH | − | 0 | + |
| 857 | 3GH | + | 0 | + |
| 858 | 3GH | − | 0 | − |
| 860 | 3GH | + | 0 | + |
| 869 | 3GH | + | 0 | + |
| 873 | 3GH | − | 0 | + |
| 876 | 3GH | − | 1GH | − |
| 877 | 3GH | − | 2GH | + |
| 881 | 3GH | + | 0 | − |

[a] W-S, Warthin-Starry stain. Designations in results are as follows: HP, *H. pylori*; GH, gastric *Helicobacter* sp. (not *H. pylori* [larger spirals]); 0, no organisms seen; 1, small numbers; 2, moderate numbers; 3, large numbers.

[b] Monkeys with cultures positive for *H. pylori*.

[c] Monkeys with cultures negative for *H. pylori*.

TABLE 2. Comparison of the results of various diagnostic tests for *H. pylori*[a]

| Test | Sensitivity | Specificity | Positive PV[b] | Negative PV | Accuracy |
|---|---|---|---|---|---|
| Culture | 57 | 100 | 100 | 18 | 61 |
| Rapid urease | 100 | 0 | 91 | —[c] | 91 |
| Histologic examination | 52 | 100 | 100 | 17 | 57 |
| Pyloristat ELISA | 14 | 100 | 100 | 10 | 22 |
| Rhesus monkey ELISA | 90 | 100 | 100 | 50 | 91 |

[a] Values are calculated by using PCR results as the reference standard.
[b] PV, predictive value.
[c] —, could not be calculated.

**Histopathology.** Warthin-Starry-stained sections were positive for *H. pylori* for 11 of the 23 animals (48%). These 11 animals were also positive by PCR (Table 1). *H. pylori* organisms were observed as curved to spiral-shaped rods, 3 to 5 μm by 0.5 μm. The sensitivity of histologic examination was 52%, and the specificity was 100%. The positive predictive value was 100%, the negative predictive value was 17%, and the accuracy was 57%. Larger gastric *Helicobacter*-like organisms, observed as spiral-shaped organisms 6 to 10 μm by 0.5 μm, were noted histologically in all animals.

**ELISA testing.** The Pyloristat ELISA test, which utilized human-based reagents, yielded positive results for only three animals, all of which were positive by PCR. The other 18 PCR-positive monkeys and the 2 PCR-negative animals were negative by the Pyloristat ELISA. The sensitivity of the Pyloristat ELISA was 14%, and the specificity was 100%. The positive predictive value was 100%, the negative predictive value was 10%, and the accuracy was 22%.

The ELISA test using homologous antigen and an anti-rhesus monkey antibody conjugate yielded results greater than or equal to 0.7 for 19 animals, all of which were PCR positive. Results of 0.5 or less were obtained for four animals, of which two were PCR positive and two were PCR negative. Because of these results, an ELISA value of greater than or equal to 0.7 was designated positive and a value of less than or equal to 0.5 was considered negative. The sensitivity of this ELISA test was 90%, and the specificity was 100%. The positive predictive value was 100%, the negative predictive value was 50%, and the accuracy was 91%.

## DISCUSSION

Fifteen years have elapsed since the initial discovery of *H. pylori* by Marshall and Warren (16). However, despite considerable effort during that period, an effective antimicrobial monotherapy or vaccine against *H. pylori* has not been developed. Because of the organism's pathogenic potential in humans, development of a more effective therapy is needed. Domestic cats and nonhuman primates are the only animals in which *H. pylori* has been found to occur naturally (12). The rhesus monkey has been proposed as an animal model of *H. pylori* for treatment and vaccine trials (1, 4, 6, 7, 18, 19, 23). Long-term studies using this model will require accurate methods of detection of *H. pylori*, including serologic testing.

Early methods of diagnosing *H. pylori* in humans relied on endoscopic collection of gastric biopsies and tissue analysis, which included rapid urease testing, cytologic evaluation of impression smears, histologic examination, culture, and, more recently, PCR. In this study, PCR testing proved to be the most sensitive method of detecting *H. pylori*, yielding positive results for 91% of the animals. Although there is a possibility of false-positive tests with PCR, the selection of specific PCR primers minimizes this chance. One limitation of PCR is that it doesn't verify the organism's viability.

Culture, although specific for *H. pylori*, was not sensitive, detecting *H. pylori* in only 12 of the 21 animals positive by PCR. Studies relying on culture as the sole diagnostic method may underestimate the prevalence of *H. pylori* in rhesus monkeys.

Rapid urease testing cannot be relied on to accurately diagnose *H. pylori* in rhesus monkeys because of the presence of other urease-positive gastric *Helicobacter*-like organisms in these animals. This was evidenced by the rapid urease tests being positive for all monkeys surveyed in this study.

Histologic examination was positive for *H. pylori* for 11 of the 21 PCR-positive animals. These animals were those in which large numbers of organisms were present, as verified by positive *H. pylori* culture. Interestingly, these were also the monkeys with the most severe gastritis (unpublished data). However, histologic examination lacked sensitivity, as 10 of the animals which were PCR positive were histologically negative for *H. pylori*.

The Pyloristat ELISA test performed suboptimally for detecting *H. pylori* in this rhesus monkey colony. Only 3 of the 21 PCR-positive animals had positive results by the Pyloristat test, yielding low values of sensitivity, negative predictive value, and accuracy. Reducing the cutoff value necessary for a positive test result did not improve the accuracy of this ELISA test. However, the ELISA test utilizing homologous rhesus *H. pylori* antigen and an anti-rhesus antibody conjugate performed well in diagnosing *H. pylori* in this study. The sensitivity and specificity of the modified ELISA were 90% and 100%, respectively, and the accuracy was 91%.

Serology is considered an accurate and reliable diagnostic test for *H. pylori* in humans (25). It has the advantage of being noninvasive compared to endoscopy and collection of gastric biopsy samples. Also, because of the patchy distribution of *H. pylori*, serology is likely to be more accurate than analysis of gastric biopsies, which evaluate only a small portion of the stomach (25). In one study, the Pyloristat test was found to have a sensitivity of 96%, a specificity of 94%, a positive predictive value of 90%, a negative predictive value of 98%, and an accuracy of 95% in humans (25).

Several studies have utilized ELISA to diagnose *H. pylori* in rhesus monkeys. One study found increased antibody levels to *H. pylori* acid extract antigen in 13 of 23 monkeys tested, although the actual *H. pylori* status of the animals was not determined by other methods (1). The study utilized human origin *H. pylori* antigen and an anti-human antibody conjugate (1). Another study discovered significantly higher antibody levels in monkeys with *H. pylori*-associated gastritis than in histologically normal animals, although the IgG antibody levels in the monkeys were only 28% of those observed in infected humans (17). The study utilized both human and rhesus monkey origin *H. pylori* strains as antigen, but used an anti-human antibody conjugate (17). In a recent article, the Pyloristat ELISA was reported to have a sensitivity of 60%, a specificity of 71%, and an accuracy of 68% for diagnosing *H. pylori* in a rhesus monkey colony (13).

In another rhesus monkey colony survey, the ELISA was found to have a sensitivity of 85% and a specificity of 92% (4). The accuracy of this ELISA was achieved by using pooled antigens from five *H. pylori* strains of human origin and an anti-rhesus monkey conjugate (4). When an anti-human antibody conjugate was substituted for the anti-rhesus antibody conjugate, a sensitivity of 69% and a specificity of 83% were noted (4). IgG optical density ratios of greater than or equal to

0.6 were considered to be positive, and optical density ratios lower than 0.6 were considered to be negative (4). The same investigators also reported positive ELISA tests for 50 to 93% of rhesus monkeys, depending on age and housing conditions, by using an antigen consisting of sonicates of *H. pylori* strains of human origin and an anti-human antibody conjugate (5). The *H. pylori* status of these animals was not determined by other diagnostic methods, however (5).

One explanation for the differing performances of the two ELISA tests in our study could be partial failure of the anti-human antibody conjugate in the Pyloristat kit to detect rhesus monkey IgG antibodies. Alternatively, the suboptimal sensitivity of the human-based ELISA may be due to *H. pylori* antigenic strain differences between human and rhesus monkey isolates. Indeed, investigators in Thailand found that an ELISA test which utilized antigens from *H. pylori* strains isolated from Thai patients had higher sensitivity, specificity, and negative predictive value than the Pyloristat test for the diagnosis of *H. pylori* in the native Thai population (2).

In conclusion, rapid urease testing was not valuable for diagnosing *H. pylori* in this colony of rhesus monkeys because of its lack of specificity. Histologic examination and culture were very specific for *H. pylori* diagnosis but lacked sensitivity. The human-based, commercially available ELISA test was also insensitive for diagnosing *H. pylori*, whereas the ELISA test utilizing rhesus monkey components correlated most accurately with the PCR results, with an accuracy of 91%. ELISA testing performed on rhesus monkeys should utilize rhesus monkey components to achieve optimal results. PCR analysis and ELISA testing appear to be important methods in diagnosing *H. pylori* in rhesus monkeys and should prove valuable in selecting and monitoring rhesus monkeys for clinical trials or longitudinal pathogenesis studies.

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