Evaluation of an Immunofluorescence Assay for Specific Detection of Immunoglobulin G Antibodies Directed against *Helicobacter pylori*, and Antigenic Cross-Reactivity between *H. pylori* and *Campylobacter jejuni*

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An immunofluorescence assay (IFA) for the detection of immunoglobulin G antibodies directed against *Helicobacter pylori* was evaluated by comparing 20 serum specimens from patients with a positive urease test on biopsy material and 20 serum specimens from patients with a negative test and with defined clinical symptoms. The resulting anti-*H. pylori* titers were classified as follows: negative, ≤64; borderline, 128; and positive, ≥256. By using these criteria, the IFA was subsequently tested, using 100 serum specimens from patients with gastric complaints. Overall, the titers were 71% positive, 10% borderline, and 19% negative. Depending on the sera's biopsy urease test results, the sensitivity and specificity of the assay were calculated to be 96%. Furthermore, these sera were classified into three subgroups on the basis of clinical manifestations: gastritis with 74% positive and 10% borderline titers, duodenal ulcer with 84% positive and 4% borderline titers, and gastric ulcer with 52% positive and 16% borderline titers. A serologic follow-up study was carried out with three patients with gastric ulcers who had been treated with colloidal bismuth subcitrate for 4 weeks and erithromycin for the final 2 weeks. The results indicate that a significant decrease in titer could be expected within 9 to 12 months after successful therapy, as determined by repeated negative CLO tests. Absorption experiments demonstrated that possible cross-reactivity between *H. pylori* and *C. jejuni* did not influence serodiagnosis.

Warren and Marshall (30) first described the association between infection with *Campylobacter pylori*, now renamed *Helicobacter pylori* (8), and the presence of type B gastritis or peptic ulcer disease. Subsequently, there has been controversy about the etiologic role of this organism in gastrointestinal disease. Recent studies have demonstrated that *H. pylori* infections induce pathogenic gastrointestinal effects in gnotobiotic piglets (13, 15), as well as type B gastritis in humans. The latter association was described by Marshall (17) and Morris (20) after ingestion of an *H. pylori* strain. As previously reported (4, 24), a highly significant correlation exists between the activity of gastritis and the grade of *H. pylori* colonization. Therefore, *H. pylori* was considered to cause type B gastritis, but its role in peptic ulceration remains less clear (2, 4, 5, 7, 22). There is continuing uncertainty concerning the mechanism of pathogenicity and the reasons for this organism's predilection for the colonization of gastric mucosa.

At the present time, the diagnosis of infections with *H. pylori* is mainly based on direct microscopy and culture from gastric biopsy specimens after endoscopy or on indirect methods based on urease activity such as the [13C]- or [14C]urea breath test or the CLO test (1, 10, 11, 18, 25). In contrast to these invasive and technically demanding diagnostic procedures, the specific detection of serum antibodies directed against *H. pylori* would be a sensitive and relatively simple tool for the evaluation of suspected infections (11, 22).

To date, serologic tests such as enzyme-linked immunosorbent assay (ELISA), immunoblotting, and complement fixation methods (2, 5, 9, 22, 27–29), which may replace direct methods when endoscopy is contraindicated, have been described. In this paper, an indirect immunofluorescence assay (IFA) is described which could be employed routinely for the detection of immunoglobulin G (IgG) antibodies directed against *H. pylori*. Possible cross-reactivity with *Campylobacter jejuni* was evaluated to assess the specificity of the IFA results.

**MATERIALS AND METHODS**

Antigenic material was produced by using *H. pylori* ERI 1, isolated in our laboratory in 1988 from a biopsy specimen obtained via gastroscopy from a 44-year-old male patient suffering from a gastric ulcer. This strain has not yet been described in the literature and will be made available. Identification of bacteria was performed both by morphological studies of bacterial cells by Gram stain and by enzymatic differentiation measuring positive urease activity, oxidase activity, and catalase activity and negative hippurate hydrolysis.

*H. pylori* was grown on sheep blood agar (4.2% Columbia agar base [Merck, Darmstadt, Federal Republic of Germany] supplemented with 1 vial of IsoVitalex [BBL Microbiology Systems, Cockeysville, Md.] per liter) at 37°C under microaerophilic conditions (CampyPak; BBL Microbiology Systems). Cells can be frozen at −70°C and recultured several months later by using a broth containing 2% tryptic soy broth, 10% glycerol, and 1% horse serum. Preparation of cells. Colonies were harvested after 3 days with 2 ml of phosphate-buffered saline (PBS) plus 0.1% NaN3 per petri dish. The bacterial cells obtained were centrifuged twice at 4°C, 10,000 × g for 15 min in the buffer
system described above. The purified material can be stored refrigerated or frozen at -70°C.

**Preparation of slides.** The working dilution of the bacterial suspension was determined by performing a series of dilution steps ranging from 1:20 to 1:320. The working dilution was the dilution which yielded approximately 50 cells per field at a 400-fold magnification with a dark-field microscope (Leitz, Wetzlar, Federal Republic of Germany). Ten microliters of this diluted suspension was placed in each well of an IFA slide (24 by 76 mm, 12 wells; Behring). The slide preparations were dried without any fixation procedures at room temperature and stored at -20°C until use.

**Performance of IFA.** IFA slides were thawed, air dried, washed for 5 min in PBS with slight stirring, and subsequently dipped into distilled water. Samples (15 μl) of patients' serum, diluted from 1:32 to 1:1,024 was applied after air drying. The slides were then incubated for 30 min at 37°C. Subsequently, they were rinsed in 10 ml of PBS, washed in PBS for 15 min, dipped again in distilled water, and then air dried. In the following step, fluorescein isothiocyanate-conjugated anti-human IgG (IgG conjugate; Dakopatts, Hamburg, Federal Republic of Germany) (10 μl per well) was applied (diluted 1:50 in PBS plus 2% fetal calf serum). The slides were incubated at 37°C for 30 min. The second-stage washing, rinsing, dipping, and drying steps were carried out as described above. A small drop of mounting fluid (PBS diluted 10:1 in glycerol) was placed in each well, and the slides were subsequently covered with glass cover slips and microscopically examined.

The fluorescence of the bacterial cells was visualized at 400-fold magnification with a fluorescence microscope (IM-405; Zeiss, Oberkochen, Federal Republic of Germany). The titer of IgG antibodies directed against *H. pylori* was determined by the reciprocal of the highest dilution of serum at which a 3+ bright, apple-green staining could be detected.

**Origin of serum specimens.** The IFA presented herein was evaluated by ascertaining titers of serum specimens from 20 patients with positive biopsy urease tests (CLO test; Heipha, Heidelberg, Federal Republic of Germany) in the presence of gastric or duodenal ulcers (group 1a). These patients would be seropositive unless they failed to generate an immunological response because the CLO test is 100% specific (1, 3, 25, 26). For controls, 20 serum samples from patients undergoing at least 5 CLO test-negative antral biopsies and exhibiting neither active antral gastritis nor peptic ulcerations (group 1b), which should therefore be seronegative to *H. pylori*, were also examined by IFA.

The diagnostic utility of the test criteria was validated by determining 100 titers in patients with clinical gastrointestinal illness who all underwent gastroscopy and CLO tests (group 2). Group 2 patients were further separated into three clinical syndromes: gastritis (group 2a), duodenal ulcer (group 2b), and gastric ulcer (group 2c). Cross-reactivity between *H. pylori* and *C. jejuni* was tested by determining the *H. pylori* IFA titers of 50 patients with positive or borderline IgG antibody reaction to *C. jejuni* as previously determined by complement fixation. Additionally, the *C. jejuni* complement fixation titers of 50 *H. pylori* IFA-positive or borderline sera were determined.

**Complement fixation tests.** The complement fixation kit for specific detection of IgG antibodies directed against *C. jejuni* was purchased from Virion (Würzburg, Federal Republic of Germany).

**Absorption studies.** Serum antibodies directed against *H. pylori* were bound by absorption with a suspension of *H. pylori* ERI 1 containing 10^8 cells per ml, which is usually used diluted in the preparation of IFA slides. Two volumes of this suspension were added to 1 volume of patient serum, and the mixture was subsequently allowed to incubate for 30 min at 37°C with gentle shaking. Bacteria were pelleted by centrifugation at 5,000 × g for 10 min at 20°C. The *C. jejuni* titer of the resulting supernatant was determined by complement fixation. After performance of the absorption procedure, all examined *H. pylori*-positive sera showed negative titers of ≤64 in the *H. pylori* IFA.

Serum antibodies directed against *C. jejuni* were eliminated by absorption with soluble antigen which was usually used as antigenic material for the *C. jejuni* complement fixation test. Two volumes of an antigen solution containing 700 μg of protein per ml were added to 1 volume of patients' serum. After incubation for 30 min at 37°C with gentle shaking, the mixture was centrifuged at 20,000 × g for 10 min at 4°C. Control experiments showed that in all cases the resulting supernatant showed negative titers after the *C. jejuni* complement fixation test was performed.

**Gel electrophoresis.** Sodium dodecyl sulfate–polyacrylamide gradient (5 to 20%) slab gel electrophoresis was carried out according to the method described by Laemmli (14). The suspension of bacteria obtained for the preparation of the IFA slides was extracted as described by Pearson et al. (23) to isolate the outer membrane proteins for further analysis. Electrophoresis was carried out with a constant current of 20 mA at 4°C in a horizontal electrophoresis chamber (Multiphor; Pharmacia, Bromma, Sweden). The gel was previously polymerized covalently on gel bond film (Serva, Heidelberg, Federal Republic of Germany) and stained with Coomassie brilliant blue R-250 (14). Statistical comparisons are based on the t test.

**RESULTS**

The morphology of *H. pylori* cells at 1,000-fold magnification under a fluorescence microscope is shown in Fig. 1. Fluorescence-labeled bacteria appeared as curved, rod-shaped cells with two to three coils. Coccoid reduction forms were detected only rarely, and no flagella were seen by using these experimental methods. Gel electrophoretic analysis of the outer membrane proteins demonstrated that *H. pylori* ERI 1 is antigenically highly similar to *H. pylori* type strain NCTC 11637 (Fig. 2).

Under the present conditions, in the group of patients (group 1) with a positive biopsy urease test result, 3 serum specimens (15%) with a titer of 128, 11 serum specimens (55%) with a titer of 256, 5 serum specimens (25%) with a titer of 512, and 1 serum specimen (5%) with a titer of ≥512 were found. Similarly, in patients with a negative biopsy urease test result, 11 serum specimens (55%) exhibited a titer of ≤32, 5 serum specimens (25%) exhibited a titer of 64, and 4 serum samples (20%) exhibited a titer of 128. Because of an overlapping in both reference groups at a serum titration endpoint of 128, negative titers were defined to be ≤64, whereas a titer of 128 was considered to be borderline and titers of ≥256 were considered presumptive evidence of an *H. pylori* infection at an undetermined time in the past.

The distribution of negative, borderline, and positive sera within the three different clinical groups is depicted in Fig. 3. Overall, group 2 produced 71% positive and 10% borderline titers, whereas within group 2a 74% were positive and 10% had borderline results. In group 2b, the greatest number of positive titers was observed, with 84% positive and 4% borderline serum samples. Positive titers of ≥1,024 occurred most frequently in this group. Patients in group 2c showed
samples known to be positive or borderline for C. jejuni, 28% were positive, 10% were borderline, and 62% were negative. When C. jejuni titers were measured for known H. pylori-positive or borderline serum samples, 2% were positive, 4% were borderline, and 94% were negative (Table 1). After absorption experiments were performed, no H. pylori-positive and only 4% borderline results could be obtained among C. jejuni-positive or borderline sera, while neither positive nor borderline results were found when determining C. jejuni titers of H. pylori-positive or borderline sera. These findings support the view that all examined H. pylori IFA and C. jejuni complement fixation test-positive patients were infected with both C. jejuni and H. pylori, rather than indicating cross-reactivity.

Table 2 illustrates the serologic changes that may ensue when complete eradication of H. pylori is achieved after treatment with colloidal bismuth subcitrate over a period of 4 weeks and erythromycin for the final 2 weeks. A slight, but measurable decrease of the anti-H. pylori IgG titer occurred 6 months after therapy, whereas a significant drop of values could be found within 9 to 12 months after successful

<table>
<thead>
<tr>
<th>Test and serum samples</th>
<th>Positive (n)</th>
<th>Borderline (n)</th>
<th>Negative (n)</th>
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<tr>
<td><strong>H. pylori IFA</strong></td>
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<tr>
<td>C. jejuni borderline (n = 10)</td>
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<td>2 (20)</td>
<td>2 (20)</td>
<td>6 (60)</td>
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<td>Postabsorption</td>
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<td>0 (0)</td>
<td>10 (100)</td>
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<td>C. jejuni positive (n = 40)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Preabsorption</td>
<td>12 (30)</td>
<td>3 (7.5)</td>
<td>25 (62.5)</td>
</tr>
<tr>
<td>Postabsorption</td>
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<td>2 (5)</td>
<td>48 (95)</td>
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<td><strong>C. jejuni CF</strong></td>
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<td>H. pylori borderline (n = 18)</td>
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<tr>
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<td>18 (100)</td>
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<tr>
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</tr>
<tr>
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<td>2 (6)</td>
<td>29 (91)</td>
</tr>
<tr>
<td>Postabsorption</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>32 (100)</td>
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treatment. In two patients, antibodies to *H. pylori* persisted for at least 1 year.

**DISCUSSION**

An IFA for detection of IgG antibodies against *H. pylori* was evaluated. This diagnostic system can be employed as a single method to provide serologic evidence of infection or as a tool to confirm positive or borderline results previously determined by ELISA or complement fixation procedures.

Serum antibodies are believed to react with strongest immunological affinity against intact bacterial cell walls when used as the antigenic source in the described IFA. The mixture of solubilized exoproteins, extracted with different methods and used in order to carry out ELISA or complement fixation, may result in decreased affinity or increased cross-reactivity (11). On the other hand, IFA titer determination often turns out to be difficult and therefore requires practical experience. A control serum with a previously determined titer of 512 was employed as a positive control in order to minimize incorrect results.

The fluorescence-labeled *H. pylori* cells were characteristically curved, rod-shaped bacteria with two to three coils after applying highly positive sera. Although no flagella were demonstrated, it is known that a species-specific flagellin of *H. pylori* in the molecular mass range of 51,000 to 62,000 Da exists (6, 16, 21) and presumably could cross-react with *C. jejuni* flagellin (21). Although gel electrophoretic analyses of the exoproteins of *H. pylori* ER1 showed high concordance with those of *H. pylori* type strain NCTC 11637 and other strains previously investigated (23, 24, 27, 30), genetic mutations that change primary and tertiary protein structures were not detectable by gel electrophoresis. These may result in various degrees of antigenicity, possibly due to variations of epitopes presented to the antibody.

The fraction of *H. pylori*-positive patients found in groups 2a, 2b, and 2c was comparable with the findings of other investigators (2, 23, 30) based on nonserologic or serologic methods. Positive anti-*H. pylori* titers of ≥1,024 occurred most frequently in group 2b. Considering these results, we conclude that *H. pylori* infection, in conjunction with a clinical manifestation like duodenal ulcer, induces a very strong systemic antibody response against *H. pylori*.

Large numbers of *C. jejuni* complement fixation test-positive or borderline sera showed cross-reactivity after *H. pylori* IFA was performed. Either approximately one-third of all cases exhibited massive cross-reactivity induced by interspecific exoproteins with identical or similar epitopes, or these patients were infected with both *C. jejuni* and *H. pylori* and therefore possess IgG antibodies against both these bacteria. Absorption experiments made clear that cross-reactivity between these two bacteria does not affect the results, since a double infection could be presumed in all cases of simultaneously *H. pylori* and *C. jejuni*-positive titers. These results support the findings of other investigators who generally consider the cross-reactivity between *H. pylori* and *C. jejuni* in serodiagnosis (11, 21, 22) to be of minor significance.

Serologic tests for *H. pylori* will be useful for monitoring the long-term outcome of therapeutic regimens aimed at eradication of *H. pylori*. On the other hand, serologic tests for *H. pylori* may also be effectively employed for seroepidemiologic studies (12) and screening of patients suffering from gastritis or peptic lesions if endoscopy is contraindicated and for certain high-risk patients before and after renal transplantation as described by von Wulffen et al. (28).

While *C. jejuni* infections are usually transmitted by contaminated food and drinking water, little has been learned about the transmission mode and the natural reservoirs of *H. pylori*. Nevertheless, Mitchell et al. (19) reported increasing incidence of *H. pylori* infections in gastroenterologists, thus supporting further the evidence of person-to-person transmission in workers using endoscopy. Further investigation of the organism reservoirs and modes of transmission should lead to a better understanding of the etiologic significance of *H. pylori* infection.

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**REFERENCES**


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