Indirect Immunofluorescence Test and Enzyme-Linked Immunosorbent Assay for Detection of Campylobacter pylori

E. SCHABER,1* F. UMLAUFT,1 G. STÖFFLER,1 F. AIGNER,2 B. PAULWEBER,3 AND F. SANDHOFER1

Institute of Microbiology, Medical School,1 and First Department of Surgery,2 University of Innsbruck, Innsbruck, and First Department of Medicine, Landeskrankenanstalten Salzburg, Salzburg,3 Austria

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An indirect immunofluorescence test (IIF) has been developed for detecting Campylobacter pylori in gastroduodenal biopsies. This test was compared with standard methods of C. pylori diagnosis, namely Gram staining and urease test, in a study population of 226 patients; 121 of the biopsy specimens were cultured for C. pylori as well. C. pylori colonization was detected in 154 of 226 patients (68%) by at least one of these methods (IIF, 96%; Gram staining, 78%; urease test, 60%; cultivation, 55%). Serum samples from 191 patients of the study population were screened for circulating antibodies to C. pylori by an indirect enzyme-linked immunosorbent assay with whole, untreated bacteria as antigen. Of these serum specimens, 140 (73%) revealed absorbance readings above the limit of positivity, which was determined as an optical density of >0.35 at 405/620 nm. Of 132 serum specimens, 128 (97%) from patients with C. pylori detected in biopsies, but only 12 (20%) of 59 specimens from those without C. pylori detection showed elevated specific antibody levels. Our data revealed that IIF proved to be the superior rapid, sensitive, and specific diagnostic method. The correlation between microbiological findings and the immune response favors our enzyme-linked immunosorbent assay as an additional tool in C. pylori diagnosis.

Since Warren and Marshall reported the detection and isolation of gastric mucosa-associated bacteria in 1983 (23), the role of Campylobacter pylori (7, 11) in the pathogenesis of gastroduodenal lesions has been under discussion. C. pylori is found in biopsies from more than 50% of patients undergoing gastroduodenal endoscopy (1, 17). An association between C. pylori colonization of the gastric mucosa and active chronic gastritis as well as duodenal ulcers has been repeatedly demonstrated (2, 4, 10, 14, 17). There is, however, considerable disagreement about a possible causal relationship between C. pylori colonization and these gastroduodenal lesions. The theory that C. pylori colonization is merely a consequence of damage of the gastroduodenal mucosa by other factors cannot be definitely excluded. The observation of Marshall et al. (15) that ingestion of about 106 C. pylori cells by a volunteer caused a histologically verified acute antral gastritis argues against this hypothesis. It has been suggested that C. pylori colonization and subsequently rapid hydrolysis of urea at intercellular junctions results in an increased back-diffusion of hydrogen ions into mucosal cells, thus predisposing them for ulcer formation (8). Recently, evidence that C. pylori produces an extracellular protease which is able to destroy the gastric mucus layer has been presented (22). This mucus layer is considered of great importance for the protection of the gastric mucosa against ulcer formation. In addition, in a follow-up study it has been demonstrated that the relapse rate of duodenal ulcers dropped significantly after complete eradication of C. pylori colonization by treatment with colloidal bismuth subcitrate (3).

Cultivation, morphological identification in smears by Gram staining, and the determination of extracellular urease are the accepted methods for detection of C. pylori in biopsy specimens of the gastric or duodenal mucosa (5, 9, 13, 18, 19). We adapted an indirect immunofluorescence (IIF) technique for the routine detection of C. pylori in mucosal biopsies. Humoral immune response to C. pylori has been assessed by agglutination, by complement fixation technique, and by enzyme-linked immunoassay (ELISA) (1, 6, 12, 20). Most of these tests are hampered by low sensitivity and lack of specificity because of cross-reactions with other bacteria (6, 12, 20), which could be overcome by using an ELISA based on whole, untreated C. pylori cells as an antigen, as was done in this study.

MATERIALS AND METHODS

Study population. A total of 101 female and 125 male consecutive patients at the First Department of Surgery, University of Innsbruck (121 patients), and the First Department of Medicine, Landeskrankenanstalten Salzburg (105 patients), undergoing diagnostic endoscopy of the upper gastroduodenal tract were examined. The mean age was 51 years (range, 8 to 95 years). The main reason for this examination was the evaluation of upper abdominal pain. Biopsy specimens from duodenum, antrum, and corpus were obtained from each patient and used for mucosal smears for IIF and Gram staining and for urease test. Only biopsies from the First Department of Surgery, Innsbruck, were investigated for viable bacteria by cultivation, since for this purpose biopsies had to arrive within 2 h at the microbiological laboratory (7).

Culture conditions. Biopsy specimens were transported to the bacteriological laboratory within 2 h in brain heart infusion broth (Oxoid, Wesel, Federal Republic of Germany). Biopsies were plated directly on fresh chocolate agar (brain heart agar; Oxoid) supplemented with 10% lysed blood and were incubated for at least 5 days in anaerobic jars in a microaerophilic environment at 37°C. Macroscopically C. pylori-like colonies were identified by Gram staining and urease test.

Gram staining. Air-dried and heat-fixed mucosal smears were stained. At least 15 min was spent in the search for gram-negative curved bacteria with typical morphology and posture.

* Corresponding author.
**Urease test.** Extracellular urease produced by *C. pylori* was detected in mucosal biopsies on agar containing 40% urea and phenol red (13). The change of color from yellow to dark pink was observed at reading times of 1/2, 2, and 6 h after inoculation of the agar plates with biopsy material. A change of color within 6 h was considered a positive result.

**Indirect immunofluorescence test.** Specific antiserum to *C. pylori* was obtained from sheep by immunization with bacteria from pooled isolates from 14 patients. Preimmune serum was secured. One sheep was immunized intravenously on days 0, 7, and 14 with 3 x 10^8 whole *C. pylori* cells, and antiserum was taken on day 21. Anti-sheep immunoglobulin G (from rabbit) was labeled with fluorescein isothiocyanate and served as the second antibody. The fluorochrome/protein ratio was determined at 495/280 nm (DU 65 Spectrophotometer; Beckman Instruments Inc., Fullerton, Calif.).

Specificity of the immune serum was assessed by two methods. First, immune serum was tested on microscopic slides coated with a suspension of freshly cultivated *C. pylori*, *Campylobacter jejuni*, *Campylobacter fetus*, *Escherichia coli*, and *W. recta* (10^11 bacteria per ml of serum; incubated for 1 h at 37°C and overnight at 4°C) (16). Working dilutions of antiserum and conjugate were determined by chessboard titration. At a dilution of 1:80 for the conjugate, the immune serum before and after absorption with bacteria related to *C. pylori* revealed a titer of 1:800, whereas the preimmune serum and the immune serum absorbed with *C. pylori* did not react with *C. pylori*. The immune serum did not show any cross-reactivity with the other bacteria. Mucosal smears were air dried and methanol fixed (10 min). Incubation and washing procedures were carried out as described by Wick et al. (24).

Nonspecific background fluorescence could be eliminated by addition of 3% bovine serum albumin to the serum dilution and 0.001% Evan blue to the conjugate. The smears were examined by using a fluorescence microscope (BH-2; Olympus Optical, Tokyo, Japan) at a magnification of x1,000. Slides were examined for 5 min.

**ELISA.** The development of an indirect ELISA to quantify circulating antibodies to *C. pylori* is described in detail elsewhere (F. Umlauf, E. Schaber, G. Stöffer, B. Paulweber, F. Sandhofer, manuscript in preparation). In brief, 5 x 10^6 whole, untreated bacterial cells per ml (*C. pylori* CCUG 15818 from the Culture Collection, University of Goeteborg, Sweden), diluted in coating buffer (0.1 M Na_2CO_3 and NaHCO_3, pH 9.6), were allowed to adhere to F-form microdilution plates (Institute Virion, Cham, Switzerland) overnight at 4°C. Additional binding sites were saturated with 0.2% gelatin. Plates were subsequently incubated with patient sera diluted 1:200 with phosphate-buffered saline (20 mM, pH 7.4) containing 0.1% Tween 20 and 0.2% gelatin and assayed in 3 wells per serum sample. Alkaline phosphatase-conjugated anti-human immunoglobulin (Sigma Chemie, Taufenkirchen, Federal Republic of Germany) was used at a dilution of 1:350. Antibody binding was estimated by reactivity with p-nitrophenyl phosphate and quantified by optical density measurement at 405/620 nm (ELISA reader, Dynatech MR 580; Dynatech, Denkendorf, Federal Republic of Germany). Two positive and one negative control serum samples were tested on each plate and served as calibration and quality controls. Specificity of the ELISA was assessed by absorption of 13 serum specimens with freshly cultivated cells of *C. pylori*, *C. jejuni*, *C. fetus*, *E. coli*, and *W. recta* (16). Anti-*C. pylori* immunoglobulin was measured before and after incubation with these bacteria. Absorbance readings dropped significantly only after incubation with *C. pylori*, regardless of whether serum samples were from patients colonized or not colonized by *C. pylori*. Two examples are shown in Fig. 1. The limit of positivity of the assay (optical density = 0.35 at 405/620 nm) was defined as the mean absorbance value of the 13 serum samples mentioned above after specific absorption of antibodies plus 3 standard deviations (16). Student’s *t* test was used to test for statistical significance of the difference of the means of the absorbance values.

**RESULTS**

**Detection of *C. pylori* in gastrooduodenal biopsies.** We investigated biopsies from 226 patients by IIF technique, Gram staining, and urease test. *C. pylori* was detected in 154 of 226 patients (68%) by at least one of the methods used. Examination of mucosal smears by IIF provided 148 (96%) positive results, whereas Gram staining yielded 120 (78%) and determination of urease yielded 92 (60%) positive findings. In only 6 cases was *C. pylori* colonization assessed by Gram staining alone (4 cases) or Gram staining and urease test (2 cases). In 23 patients, *C. pylori* detection was obtained exclusively by IIF (data not shown). IIF proved to be the most sensitive single diagnostic procedure. Of the biopsies, 121 were also cultured for *C. pylori*. In 86 of these samples, *C. pylori* detection succeeded by one of the four techniques used, in 47 cases (55%) by culture (Fig. 2).
Circulating antibodies of C. pylori. Absorbance readings of 191 serum specimens from patients with and without proven C. pylori colonization are shown in Fig. 3. Of these specimens, 140 (73%) revealed elevated antibody titers. A total of 128 of 132 (97%) serum samples from patients with positive C. pylori findings in biopsies and 12 of 59 (20%) sera from those without C. pylori detection gave positive absorbance readings. Also, the mean absorbance reading of sera from the C. pylori-positive population (optical density = 0.86 ± 0.3) was significantly higher than that of sera from the C. pylori-negative population (optical density = 0.32 ± 0.25) (P < 0.001).

Relation between endoscopic diagnosis, C. pylori detection in biopsies, and ELISA results. Our results are in good accordance with those of other authors (1, 6, 17). A strong correlation between diagnoses of ulcus duodeni, gastritis, duode(n)itis, and to a lesser extent ulcer ventriculi and the detection of C. pylori in biopsies and anti-C. pylori immunoglobulin by ELISA was found (Table 1).

**DISCUSSION**

For routine diagnosis of C. pylori colonization, methods such as Gram staining, urease test and cultivation have been used so far. Each of these methods has considerable disadvantages with regard to specificity and sensitivity. We compared our IIF test with these other diagnostic techniques by investigating specimens from 226 consecutive patients undergoing endoscopy of the upper gastrointestinal tract. Cultivation is known to be specific but has some disadvantages. C. pylori is a very delicate organism which easily loses its viability during transport (7). The relatively low isolation rate of C. pylori by culture may be related to the fact that no suitable liquid transport medium is presently available. Therefore, it is not surprising that our results suggest that cultivation is not the method of choice for routine diagnosis of C. pylori. Biochemical (urease) and microscopic (Gram staining) methods are quick, but a large number of microorganisms in the sample is necessary for a positive result.

The application of the IIF technique combines the high specificity and sensitivity of an immunological reaction with the rapidity of a microscopical examination method, as demonstrated by our results. Since the antiserum used in this IIF test showed no cross-reactivity with bacteria closely related to C. pylori, false-positive results are considered most unlikely. The low failure rate of 2.7% false-negative results by IIF can be eliminated by additional use of urease test and Gram staining.

C. pylori colonization of the gastric mucosa generally leads to systemic antibody response in the host, which has been demonstrated by different authors (1, 6, 12, 20). Most of the ELISAs specific for antibodies to C. pylori which have been published so far revealed poor specificity as a result of cross-reactivity of the antigen preparations with other bacteria. Using whole, untreated C. pylori cells, we were able to overcome this problem. Absorption experiments proved that our antigen preparation did not share antigenic determinants with other representative members of the genus Campylobacter (C. fetus and C. jejuni) or with the phylogenetically closely related bacterium W. recta (21). The latter observation is of special importance because W. recta is a resident of the dental flora in man. The serum antibodies could be absorbed only by incubation with C. pylori. Thus, elevated antibody levels have to be regarded as specific immune response to C. pylori colonization. Actually, 97% of the patients with microbiologically verified C. pylori coloniza-

### TABLE 1. Comparison of endoscopic diagnoses with detection of C. pylori in biopsies and specific antibodies in sera

<table>
<thead>
<tr>
<th>Endoscopic diagnosis</th>
<th>Biopsy</th>
<th>ELISA</th>
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<tbody>
<tr>
<td></td>
<td>No. of patients</td>
<td>No. (%) of positives</td>
</tr>
<tr>
<td>Gastritis</td>
<td>92</td>
<td>78 (85)</td>
</tr>
<tr>
<td>Duodenitis</td>
<td>29</td>
<td>23 (79)</td>
</tr>
<tr>
<td>Ulcus duodeni</td>
<td>26</td>
<td>23 (88)</td>
</tr>
<tr>
<td>Ulcus ventriculi</td>
<td>14</td>
<td>10 (71)</td>
</tr>
<tr>
<td>No abnormality</td>
<td>82</td>
<td>33 (40)</td>
</tr>
</tbody>
</table>

*Biopsies from 226 patients and sera from 191 patients were assayed; 17 patients had two diagnoses at the same time.

*Positive by at least one (IIF, Gram staining, urease test, or cultivation).
tion revealed elevated antibody levels. Therefore, ELISA results strongly support the diagnosis of \textit{C. pylori} colonization. Of patients in whom \textit{C. pylori} was not detected, 20% showed elevated antibody titers. At present, it cannot be determined whether these patients were actually colonized by \textit{C. pylori} and the bacterium had not been detected or whether they had a previous infection with no decay of serum antibodies, since it is not known how long antibodies to \textit{C. pylori} persist. Such patients should be kept under observation, and biopsies should be taken again if abdominal complaints persist. Only 3% of the \textit{C. pylori}-positive population showed very low absorbance readings, which might represent immune response very early in \textit{C. pylori} colonization.

Once more, the high detection rate of \textit{C. pylori} colonization (68%) and of specific antibodies (73%) in patients with upper abdominal complaints and the strong correlation with endoscopic diagnoses suggest that \textit{C. pylori} plays an important role in the pathogenesis of gastroduodenal lesions. The IIF technique with high specificity and sensitivity and the ELISA providing additional information are useful tools for the diagnosis of \textit{C. pylori} in patients with upper abdominal pain.

LITERATURE CITED


