Simultaneous Detection and Differentiation of Anti-\textit{Helicobacter pylori} Antibodies by Flow Microparticle Immunofluorescence Assay

F. Bühlīng,1* G. Koch,1,2 T. Wex,3 A. Heimburg,1,2 M. Vieth,4 A. Leodolter,3 A. Roessner,4 S. Ansorge,2 and P. Malfertheiner3

Institute of Immunology,1 Department of Gastroenterology,3 and Department of Pathology,4 Otto-von-Guericke University Magdeburg, and Institute of Medical Technology Magdeburg (IMTM) GmbH,2 39120 Magdeburg, Germany.

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\textit{Helicobacter pylori} is the key pathogen for gastroduodenal diseases. The clinical outcome of \textit{H. pylori} infection is influenced by the presence of strain-specific virulence factors that are usually detected by the presence of specific anti-\textit{H. pylori} antibodies in serum. Apart from the detection of these antibodies by enzyme-linked immunosorbent assay (ELISA), it is desirable to obtain additional information concerning the presence of certain virulence factors of \textit{H. pylori} that are currently detected by immunoblot analysis. At present, the immunodiagnosis of an \textit{H. pylori} infection includes two separate methods: ELISA and immunoblot analysis. Here, we report the development and evaluation of a new rapid flow microparticle immunofluorescence assay (FMIA) for detection of anti-\textit{H. pylori} antibodies in human serum. The assay allows rapid qualitative and quantitative detection of anti-\textit{H. pylori} antibodies using crude antigen preparations as well as single recombinant antigens (urease A, urease B, CagA, and alkylhydroxy peroxide reductase) in the same sample with one measurement, and thus it combines the advantages of enzyme immunoassay and Western blot analysis. Seventy-five patient samples were analyzed by FMIA, ELISA, and Western blotting with respect to their immunoreactivity against crude \textit{H. pylori} extracts and individual \textit{H. pylori} antigens. Statistical analyses revealed an overall similarity of more than 90% among the results for FMIA, ELISA, and Western blot. Therefore, we conclude that FMIA is a powerful and time- and cost-saving assay system for the detection of antimicrobial antibodies, with higher sensitivity and a larger measurement range than ELISA.

\textit{Helicobacter pylori} is a gram-negative, spiral-shaped bacterium that colonizes the gastric epithelium and predisposes to severe diseases, such as duodenal ulcer and gastric cancer. Since only a subset of infected patients develop clinically significant diseases, research has focused on identifying factors and markers that define high-risk patients in whom \textit{H. pylori} infection needs to be eradicated. In addition to host-dependent factors, a major reason for these differences is presumed to be the heterogeneity of \textit{H. pylori} strains with respect to the expression of virulence factors. Strains with a high pathogenic potential are characterized by the expression of cytotoxin-associated protein A (CagA) and vacuolating cytotoxin (VacA) (2, 9, 11). Other proteins are expressed in strains with higher or lower pathogenic potential. These antigens are urease A (UreA), urease B (UreB), alkylhydroxy peroxide reductase (APR), and flagellin (7, 8).

Serological studies have shown that the presence of antibodies against several antigens, particularly against VacA and CagA, might be related to the severity of gastroduodenal disease. Anti-CagA antibodies have been detected significantly more frequently in sera of patients with gastric cancer or duodenal ulcer than in patients with chronic gastritis or other gastroduodenal diseases (3).

Diagnostic tests for detection of \textit{H. pylori} infections in patients should be reliable, easy and quick to perform, and non-invasive. The identification of strains with high pathogenic potential will definitely support therapeutic decisions and thus decrease costs. For detection of \textit{H. pylori} infections, serological methods play an important role in clinical practice. A number of serological tests for detection of anti-\textit{H. pylori} antibodies have been developed during recent years. Serological diagnosis is usually performed as a two-step process. First, sera are screened with enzyme-linked immunosorbent assays (ELISAs), which investigate crude antigen preparations. These assays are characterized by a high sensitivity, and they are rapid and may be automated to give quantitative results. On the other hand, they do not differentiate strains with high versus low pathogenic potential. Therefore, to identify anti-CagA or anti-VacA antibodies, positive samples are analyzed in a second step by Western blotting. Western blot analyses are highly specific but time-consuming. Furthermore, they are, despite computer-supported evaluation programs, difficult to evaluate, and they do not give quantitative results.

Alternatively, \textit{H. pylori} infections can be detected by histological methods and cell culture. These methods are characterized by a high specificity, but their sensitivity is low and depends very much on the experience of the pathologist or the laboratory.

Here we report the development and evaluation of a new rapid flow microparticle immunofluorescence assay (FMIA) for detection of \textit{H. pylori} antibodies. The assay allows the fast qualitative and quantitative detection of anti-\textit{H. pylori} antibodies by using crude antigen preparations and single recombinant antigens simultaneously. Thus, it combines the values of the enzyme immunoassay (EIA) and Western blot in a single quantitative assay.
TABLE 1. Primer sequences used for cloning and expression of H. pylori antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primer direction</th>
<th>Sequence</th>
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<tr>
<td>Urease A</td>
<td>Forward</td>
<td>5′-GGAAATTTCAATGGAAAAACCTACCCCCAAAAGGTAGAC</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5′-GGAAATTTCAATGTTACTTCTTAAATGG</td>
</tr>
<tr>
<td>Urease B</td>
<td>Forward</td>
<td>5′-GGAAATTTCAATGGAAAAAGATTAGCGAAAAAGATATGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-GGAAATTTCAATGTTACTTCTTAAATGG</td>
</tr>
<tr>
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<tr>
<td></td>
<td>Reverse</td>
<td>5′-CATATGTTAGATTGTTGAAACCCAC</td>
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<tr>
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<td>Forward</td>
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</tr>
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<td></td>
<td>Reverse</td>
<td>5′-GGAAATTTCAATGTTAGTACAAAACTTGCCCAGAC</td>
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**MATERIALS AND METHODS**

**Patients.** Seventy-five patients with dyspepsia who presented at the Department of Gastroenterology were included in the study. None of these patients had received nonsteroidal anti-inflammatory drugs or proton pump inhibitors before blood was taken. Patients were included after giving their written informed consent. The *H. pylori* status of patients was analyzed by ELISA (Pyloriset EIA-G III; BAG GmbH, Lich, Germany). Sera were initially aliquoted and stored at −30°C until use. During the study, samples were subjected to two to three freeze-thaw cycles.

**Generation of H. pylori antigen extracts.** Clinical isolates of *H. pylori* were cultured under microaerophilic conditions at 37°C in brucella broth (Difco Laboratories; Detroit, Mich.) for 4 days. Bacteria were centrifuged at 6,000 × g at 4°C and resuspended in phosphate-buffered saline (PBS) or 0.1 M borate buffer (pH 8.5). Suspensions were sonicated on ice and centrifuged at 10,000 × g for 10 min. The protein concentration of the retained supernatants was measured with the Micro-BCA (bicinchoninic acid) assay (Bio-Rad, Munich, Germany).

**Generation of recombinant H. pylori antigens.** (i) Plasmid construction. Clinical isolates of *H. pylori* were cultured under microaerophilic conditions at 37°C in brucella broth for 4 days. Bacteria were pelleted by centrifugation and washed and reconstituted in Tris-EDTA buffer. *H. pylori* DNA was prepared with the Invitrogen genomic DNA kit III (Invitex, Berlin, Germany). The coding sequences of the urease A, urease B (GenBank accession no. AB032429), 26-kDa protein (GenBank accession no. AE000654 and AE000511), and CagA 50-kDa fragment (amino acids 30 to 460; QLVAF...DPKHL; GenBank accession no. AB003397) were amplified from *H. pylori* DNA by PCR. The characteristics of the primers used for amplification are given in Table 1. Add-on recognition sites for the restriction enzyme NdeI, used for cloning into the vectors, were introduced. Amplified DNA fragments were controlled by sequencing (GATC Biotech, Konstanz, Germany) and inserted into pET-1b expression vector (Novagen, Madison, Wis.), which also coded for an additional N-terminal His tag sequence that was used for purification by metal chelate chromatography.

(ii) Expression and recombinant protein purification. Plasmids were transfected in Escherichia coli BL21(DE3) pLysS expression cells (Novagen). Cells were grown in 200 ml of Luria-Bertani (LB) medium with 100 μg of ampicillin per ml at 37°C. Cultures were induced with 0.25 mM IPTG (isopropyl-β-D-thiogalactospyranoside) for 3 h at 22°C. Cells were harvested by centrifugation at 5,400 × g for 15 min at 4°C, and pellets were resuspended in extraction buffer (BugBuster; Novagen). Soluble and insoluble fractions were separated by centrifugation at 10,000 × g for 10 min at 4°C. Recombinant proteins were purified from the soluble fraction by metal chelate chromatography with Ni-nitrilotriacetic acid resin (Novagen) followed by gel filtration. Antigen preparations were controlled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 11% polyacrylamide gels and immunoblotting with a polyclonal rabbit anti-*H. pylori* serum (DAKO, Hamburg, Germany).

(iii) SDS-PAGE and immunoblotting. Antigen preparations were separated by SDS-PAGE (6). Proteins were stained with Coomasie brilliant blue R250 or were transferred to a Hybond ECL membrane (Amersham Pharmacia, Freiburg, Germany) using a Semi-dry transblot system (Bio-Rad, Munich, Germany). Filters were blocked with 1% nonfat dry milk and incubated for 1 h with 1-μg/ml anti-*H. pylori* antibody (DAKO) or 20-ng/ml anti-His tag antibody (Novagen) to identify recombinant fusion proteins. The immunoreaction was detected with a second alkaline phosphatase-labeled antibody (anti-rabbit immunoglobulin G [IgG]; Dianova, Hamburg, Germany) and nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate as a substrate.

**Histological detection of H. pylori infection.** For histology, paraffin-embedded step analysis, coated with hematoxylin and eosin to grade gastritis according to the updated Sydney system (4) and with Warthin-Starry-Silver stain to detect *H. pylori*. Histological evaluation was performed by an experienced gastrointestinal pathologist (M.V.) who was not aware of clinical history or symptoms.

**Detection of H. pylori antibodies by using commercial assay systems.** Sera were analyzed with a commercially available ELISA system (Pyloriset EIA-G III) and two Western blot assays obtained from Viramed (Munich, Germany) and Biosens (Oberhaching, Germany), referred to as Blot 1 and Blot 2, respectively. All procedures were performed according to the manufacturers’ instructions. Unless indicated otherwise, serum probes were diluted 1:75 or 1:100 for Western blots and 1:200 for ELISA.

In the ELISA, microtiter wells were coated with inactivated *H. pylori* antigen extracts and used for qualitative and quantitative antibody testing. The results were expressed in arbitrary enzyme units (EU) per milliliter of serum. They were considered positive if the number of *H. pylori* antibodies was equal to or above that of a calibrator serum (20 EU/ml), which was part of the assay system. Probes with values lower than 20 EU/ml were negative.

In both Western blot systems, defined isolates of several *H. pylori* strains were separated by SDS-PAGE and blotted on nitrocellulose membranes. The antigen preparations contained the following strain-specific proteins: CagA, VacA, 30-kDa protein, UreA, UreB, heat shock protein, and flagellin (Fig. 1A). The immunoreactions of individual patient sera were analyzed visually. They were classified positive or negative when compared with the reference strips.

The results obtained using Blot 1 were compared with those of Blot 2, which contained similar antigens (Fig. 1A) and was performed and evaluated as described above.

**Microparticle assay.** Nonfluorescent paramagnetic polystyrene microparticles (Kisker GmbH, Mülheim, Germany) with diameters of 6.5, 8, 10, and 12 μm and containing functional carboxyl groups were used. When indicated, fluorescein isothiocyanate (FITC)-labeled microparticles (8 μm) were used. Each microparticle population was coated with either *H. pylori* antigen extract or a distinct recombinant antigen by using the carboxylate kit for carboxylated microparticles (Polysciences, Inc., Warrington, Pa.). Different antigen concentrations were used for determination of optimal coating conditions, taking into consideration the amount of bound antigen and the homogeneity of coating. A concentration of 400 μg of antigen per ml was chosen for the coating of a 2.5% microparticle suspension.

For analysis, coated microparticles were mixed, and about 100,000 microparticles were incubated with 100 μl of patient serum in 96-well plates (serum dilutions of 1:200 and 1:1,000) in PBS (pH 7.4) supplemented with 5% bovine serum albumin (BSA; Sigma, Deisenhofen, Germany) at room temperature for 1 h. Next, microparticles were washed with a Promega MagneSphere 96 rack in PBS–1% BSA–0.1% Tween 20. The immunoreaction was detected with phycoerythrin-conjugated antihuman IgG (heavy and light chains) serum (Dianova).

Flow cytometric analyses were carried out with a FACSCalibur flow cytometer (BD, Heidelberg, Germany), equipped with an argon laser operating at 488 nm. Different microparticle populations were gated considering their forward scatter and their fluorescence intensity in the green channel (FL1, around 520 nm). The immunoreaction was quantified by registration of mean fluorescence intensities and peak channels of the respective microparticle populations in the red channel (FL2, around 660 nm). Results derived from the mean fluorescence intensities and the peak channel analyses showed no significant differences. Therefore, only mean fluorescence intensities are shown. The fluorescence intensity is given in
arbitrary fluorescence units (FU). Nonhomogeneous staining resulting in a skewed fluorescence distribution was found in single patient samples and did not result in significant differences between peak channels and mean fluorescence intensities.

Cutoff values for each antigen-microparticle population were calculated from the fluorescence intensities measured from a pool of at least 10 negative samples (according to commercial test systems). The cutoff value was calculated by the following formula: cutoff fluorescence intensity = mean fluorescence intensity of negative samples + (3 \times \text{standard deviation}).

**Statistical evaluation.** All statistical analyses were performed with SPSS 10.0 for Windows (SPSS, Chicago, Ill.). Bivariate correlation coefficients were calculated according to Pearson.

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**RESULTS AND DISCUSSION**

**Characterization of recombinant proteins.** Cloned *H. pylori* antigen DNA sequences in the pET-14b vector were expressed in *E. coli* cells. Figure 1B shows purified recombinant antigens from the soluble fraction in SDS-PAGE and Western blot reactions. UreA, UreB, and APR protein were produced as full-length 26-, 66-, and 22-kDa recombinant proteins. CagA was produced as an N-terminal 50-kDa fragment of the 128-kDa protein. The fragment contained all epitopes that are

![FIG. 1. Detection of antibodies against defined *H. pylori* antigens using Western blot analyses. (A) Commercially available Western blots were incubated with rabbit *H. pylori* antiserum. (B) Recombinant antigens prepared as described in Materials and Methods and separated by SDS-PAGE, blotted on nitrocellulose, and stained with rabbit *H. pylori* antiserum.](image)

![FIG. 2. Detection of anti-*H. pylori* antibodies using FMIA. Antigen-coated microparticles were prepared as described in Materials and Methods. (A) Defined microparticle populations were separated by size (forward scatter) and FITC fluorescence (around 520 nm). (B) Specific anti-*H. pylori* immunoreactivity was analyzed by using phycoerythrin-labeled anti-human IgG antibodies. The fluorescence intensity around 560 nm correlated with the concentration of anti-*H. pylori* antibodies in the serum samples. For each microparticle population, a sample that was incubated with patient serum (shaded curve) and a sample that was incubated with buffer alone (negative control, open curve) are shown.](image)
recognized by the majority of naturally occurring antibodies (5). The levels of expression were 5 to 25 mg of protein per liter of culture medium.

Characterization of the microparticle assay. To establish an assay system that combines the diagnostic value of ELISA and Western blotting, microparticle populations were coated with either crude *H. pylori* extracts or purified recombinant antigens. Then the populations were mixed in equal amounts and incubated with patient sera and phycoerythrin-labeled anti-human IgG. The immunoreactivity of single microparticles was measured by flow cytometry (Fig. 2). Populations coated with various proteins were differentiated according to their size (forward scatter) and their fluorescence in the green channel. Thus, five populations were gated (12 μm, R5; 10 μm, R4; 8 μm [with FITC], R3; 8 μm, R2; and 6.5 μm, R1). The immunoreactivity mediated by anti-*H. pylori* antibodies in the patient sera correlated with the mean fluorescence intensity in the red channel.

Comparative evaluation of microparticle assay, ELISA, and histology. A large measurement range is important for sensitive and accurate detection of high and low antibody concentrations at a given dilution. Therefore, the measurement range of FMIA was compared with that of the ELISA. Patient sera were diluted between 20- and 100,000-fold and incubated either with coated microparticles or in the ELISA plates. For the crude *H. pylori* antigen preparation, the measurement range of the FMIA was between dilutions of 100- and 64,000-fold, whereas the measurement range of the ELISA was between dilutions of 25- and 500-fold (Fig. 3). Thus, the FMIA was characterized by a higher sensitivity and larger measurement range.

A 1,000-fold serum dilution was used for further FMIA measurements, and a 200-fold dilution was used for ELISA. These serum dilutions were shown to be in the linear measurement range of the respective assays.

Furthermore, corresponding mean fluorescence intensities derived from the measurement of the *H. pylori*-coated microparticle population were correlated to the enzyme units derived from ELISA (Fig. 4). The values derived from positive sera showed a significant bivariant correlation (*r* = 0.8, *P* = 0.01).

The cutoff value (300 FU) of the FMIA was calculated as described in Materials and Methods. The cutoff value for the ELISA (20 EU) was given by the manufacturer (10). All samples below 13 EU were negative in FMIA. Only some of the samples with values between 14 and 19 EU were negative in FMIA (44%, 180 to 280 FU); most of these samples, however, were above the threshold calculated for FMIA (56%, 400 to 1,000 FU). All ELISA-positive samples (>20 EU, ranges of 20 to 900 EU) were classified as positive in FMIA (400 to 8,000 FU) (Fig. 4). Remarkably, the microparticle assay showed a wider range of mean values than the ELISA. In particular, the discrimination of samples at the borderline between positive and negative values was better in the microparticle assay than in the EIA (14 to 19 EU versus 180 to 1,000 FU). All samples with controversial results in ELISA and FMIA were investigated by Western blotting to analyze whether the immunoreactivities detected in FMIA were specifically related to distinct *H. pylori* antigens. We found that all samples that were positive in FMIA showed distinct bands in the Western blot for either UreB or CagA. From this, we concluded that the FMIA is more sensitive than the ELISA system.

Alternatively, the agreement of qualitative results between
ELISA and FMIA could be increased above 90% if the cutoff value of FMIA was increased empirically to 700 FU.

Furthermore, the FMIA and the ELISA results were compared to histological findings derived from gastric biopsy samples (Fig. 4B and C). We found that samples from all patients with positive histology were positive in FMIA and ELISA. This corresponds to the high sensitivity of both methods. On the other hand, in sera of some patients that were negative in histology, we found anti-\textit{H. pylori} antibodies in FMIA and ELISA (51 and 30%, respectively). Considering also the detection of specific bands in the Western blot, we suggest that these differences between histology and FMIA or ELISA support the lower specificity of all three methods (1). Again, the number of false-positive samples in FMIA could be decreased to a number similar to that of the ELISA by increasing the cutoff value to 700 FU.

Comparative evaluation of the microparticle assay and Western blot. Mean fluorescence intensities derived from the measurement of beads, which were coated with single recombinant \textit{H. pylori} antigens, were correlated to the semiquantitative evaluation of the Western blots (Fig. 5). FMIA results of UreA-coated beads showed an agreement rate of 87% with those derived from Blot 1. Five percent of the samples were false positive and 8% were false negative in the FMIA compared to Western blot. Immunoreactivities for UreB showed similar results: an agreement of 87%, a false-positive identification of 4%, and a false-negative identification of 10%. The highest correlation was found for CagA, with an agreement of 90% between the microparticle assay and Blot 1, whereas 6% were false positive and 4% were false negative. The lowest correlation was found for APR with 66% agreement (4% false positive and 30% false negative).

To analyze whether these differences between FMIA and blot 1 were related to the detection systems or to antigen preparations, we compared Western blots from different manufacturers. As shown in Fig. 6, the correlation between Western blots obtained from different manufacturers was similar to that of FMIA and Blot 1. Again, we found agreement rates between 87 and 90% for all antigens investigated, except APR. Thus, the differences may result from variations in the \textit{H. pylori}
antigens in Blot 1 (Viramed) and Blot 2 (Biosens). Patient samples (n = 29) were each analyzed in Blot 1 and Blot 2. The corresponding results were depicted in dot blots. Only those samples that could be clearly evaluated in both Western blots were used for comparative analyses (urease A, 23 of 29 samples; urease B, 24 of 29 samples; CagA, 26 of 29 samples; APR, 23 of 29 samples).

strains used for antigen preparation, the amounts of antigen used for coating of the microparticles, or the antigen preparation methods. In addition, the Western blots were sometimes difficult to evaluate because of faint bands.

Since the highest variation was found in the antibodies directed against the APR protein, we speculated that different assay systems contained different antigens with a similar molecular weight. This hypothesis was supported by the finding that the respective protein appeared as a single band in Blot 2 but as a double band in Blot 1. Interestingly, patient sera, which were positive in FMIA, selectively stained the upper band in Blot 1. Therefore, we suggest that the lysates that were separated to generate the Western blots contained more than one protein at a molecular mass around 22 kDa.

Seroprevalence of antibodies against defined H. pylori antigens. Recombinant H. pylori antigens were used to evaluate the specificity of antibodies in patient sera. We tested whether it was possible to assign a specific immunoreactivity to each serum that stained the microparticles coupled to the crude H. pylori extract. The highest seroprevalence was found for anti-UreA, anti-UreB, and anti-CagA antibodies. All samples from seropositive patients, retrieved in the assay using H. pylori extract, contained antibodies against either UreA or UreB; about 30% of the positive sera contained antibodies against both urease isoforms. CagA antibodies were found in 68% of the seropositive samples, while anti-APR antibodies were found only in 17% according to the microparticle assay and 43 or 67% of the patient sera according to the blot systems (Table 2).

We conclude that the recombinant antigens UreA, UreB, and CagA, and to some extent APR, as well as H. pylori whole-cell lysate, are useful antigens in microparticle assays for the detection of anti-H. pylori antibodies. Results obtained by FMIA were similar to those from commercially available ELISA and Western blot assays. Thus, FMIA indeed integrates both ELISA and Western blotting and is a time- and consequently cost-saving method for detection of antimicrobial antibodies.

ACKNOWLEDGMENT

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REFERENCES


TABLE 2. Seroprevalence of antibodies against specific H. pylori antigens in seropositive patients

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<tr>
<th>Antigen</th>
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<tr>
<td>Blot 1</td>
<td>FMIA</td>
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<tr>
<td>UreA</td>
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<td>55</td>
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<td>CagA</td>
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"n = 60."