A Novel Line Immunoassay Based on Recombinant Virulence Factors Enables Highly Specific and Sensitive Serologic Diagnosis of *Helicobacter pylori* Infection

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Helicobacter pylori colonizes half of the world's population, and infection can lead to ulcers, gastric cancer, and mucosa-associated lymphoid tissue (MALT) lymphoma. Serology is the only test applicable for large-scale, population-based screening, but current tests are hampered by a lack of sensitivity and/or specificity. Also, no serologic test allows the differentiation of type I and type II strains, which is important for predicting the clinical outcome. *H. pylori* virulence factors have been associated with disease, but direct assessment of virulence factors requires invasive methods to obtain gastric biopsy specimens. Our work aimed at the development of a highly sensitive and specific, noninvasive serologic test to detect immune responses to important *H. pylori* virulence factors. This line immunoassay system (*recom*Line) is based on recombinant proteins. For this assay, six highly immunogenic virulence factors (CagA, VacA, GroEL, gGT, HcpC, and UreA) were expressed in *Escherichia coli*, purified, and immobilized to nitrocellulose membranes to detect serological immune responses in patient's sera. For the validation of the line assay, a cohort of 500 patients was screened, of which 290 (58.0%) were *H. pylori* negative and 210 (42.0%) were positive by histology. The assay showed sensitivity and specificity of 97.6% and 96.2%, respectively, compared to histology. In direct comparison to lysate blotting and enzyme-linked immunosorbent assay (ELISA), the *recom*Line assay had increased discriminatory power. For the assessment of individual risk for gastrointestinal disease, the test must be validated in a larger and defined patient cohort. Taking the data together, the *recom*Line assay provides a valuable tool for the diagnosis of *H. pylori* infection.

Half of the world's population is infected with *Helicobacter pylori*, with an estimated prevalence of up to 90% in developing countries and an average of 30 to 40% in developed countries (1). Because of its association with ulcer disease and gastric cancer (2–5), the infection represents a major global health problem. While all infected individuals have active but subclinical gastritis (6), only a minority develop the associated diseases (7). Currently, treatment of the infection is mainly recommended for patients with ulcers or gastric cancer or who are considered at high risk for gastric cancer after definite diagnosis of the infection (8). In areas with high incidence of gastric cancer, population-based screening and treatment measures are being discussed in order to reduce the incidence of gastric cancer (9). Japan has recently revised its guidelines and now recommends screening for *H. pylori* infection and treatment of all infected individuals (10). However, cost-effectiveness analyses have shown that the successful implementation of such "test and treat" strategies is highly dependent on the reliability of the screening test applied, as well as its associated (direct and indirect) costs (11). While a lack of sensitivity will miss patients who should be treated, a lack of specificity leads to unnecessary treatment with all its consequences, such as costs, side effects, and aggravation of cross-resistance. Given the high prevalence of the infection, a hypothetical loss of specificity of 5% in a high-prevalence and high-risk population like that of China would lead to unnecessary treatment of millions of individuals. In Western countries, where endoscopic evaluation of positive patients above a certain age prior to eradication therapy is recommended by many gastroenterologists to avoid overlooking gastric

cancer, tests with low specificity would lead to considerable costs. Therefore, highly accurate tests are mandatory for the management of *H. pylori* infection in all populations. At present, a number of diagnostic tests are available. Endoscopy is still the gold standard for patients at increased risk, enabling the inspection of the gastric mucosa and biopsy sampling for histological analysis. Specific staining procedures, such as Warthin-Starry, or direct culture of *H. pylori* from gastric mucosa can identify active infection with very high specificity, although sensitivity often varies (12). Also, endoscopy is not always available and, as a costly and invasive method, is not recommended for screening approaches. Furthermore, biopsy sampling errors due to the discontinuous distribution of the infection can lead to false-negative results (13). Therefore, noninvasive tests are recommended for first-line assessment of *H. pylori* infection. Here, the [^{13}C]urea breath test (UBT), serology, and the stool antigen test offer high accuracy at

relatively low cost. UBT can further identify active, ongoing infection but depends on comedication and the compliance of the patient, as well as the availability of measuring devices. In Germany and other countries, UBT is not approved for screening of infection but is mainly recommended for evaluating successful eradication and in cases of suspicion of reinfection (14). Stool antigen tests detect *H. pylori* proteins in the stool. Different stool antigen tests using poly- or monoclonal antibodies are available. However, these test systems show relatively low sensitivities and specificities of 49 to 92% and 76 to 94%, respectively, as shown in recent studies by Calvet et al., Kesil et al., and Korkmaz et al. (15, 16, 17). While stool antigen is the preferred test for use in children (18), its reliability and predictive value in adults are lower (19). However, it has been shown that, in some clinical situations, these tests have the ability to distinguish between infected and successfully treated patients (20). Serology is one of the most widely employed methods for screening larger populations, and several test systems have been developed. Most tests are based on enzyme-linked immunosorbent assay (ELISA) formats using *H. pylori* lysates or purified antigens, like CagA and VacA (21, 22). They are cheap and easy to perform but either cannot discriminate between type I and type II strains or suffer from a lack of sensitivity, which could be due to the type or number of antigens used. In recent years, many ELISAs and solid-phase tests have reached the market, but hardly any of them have been evaluated in large trials or compared to approved systems in larger populations. Thus, there is an urgent need to develop a quick, easy, reliable, and reproducible noninvasive test for the detection of patients at high risk for disease. Here, we describe a highly sensitive and specific line immunoassay based on recombinant *H. pylori* proteins, which are known to be involved in virulence, colonization, and immune evasion. Moreover, based on the detection of individual antigens, this test might provide additional information on the virulence of the persisting *H. pylori* strain to support decisions on the appropriate treatment.

MATERIALS AND METHODS

Study population. From October 2009 to January 2012, a total of 500 patients undergoing routine upper gastroscopy for gastrointestinal complaints were recruited for this study. The patient population had a random distribution of gender, age, and origin. Patients who had undergone *H. pylori* eradication therapy, were receiving active immunosuppressive therapy, or were suffering from malignant diseases were excluded from this evaluation study. The ethics committee of the Technische Universität München, Munich, Germany, approved the study.

Selection of antigens. For the development of the new line immunoassay, nine different *H. pylori* proteins were initially considered possible candidates: CagA (CAB37821.1), VacA (Q48247), GroEL (EIE28183.1), UreA (NP_222790), UreB (NP_222789.1), gGT (YP_002266681), HcpC (YP_002301695), ICD (YP_002265666), and Omp18 (NP_207916). This selection was based on antigen properties like virulence factors (e.g., CagA, VacA, GroEL, UreA, and UreB) or immune evasion factors (e.g., gGT and VacA) or ubiquitously expressed *H. pylori* proteins (ICD and Omp18). Thus, we focused on surface-localized or secreted *H. pylori* proteins.

Cloning of antigens. The protein-coding sequences without signal peptides were amplified under standard PCR conditions from type I *H. pylori* strains (26695, G27, and J99; primer sequences are listed in Table S1 in the supplemental material) with appropriate restriction enzyme recognition sites for further subcloning into different expression plasmids (pQE2 [Qiagen], pMal [New England BioLabs], pDS1 [Addgene], pET30 [EMD Biosciences], and pUC8 [Sigma]). Correct insertion of the respective sequences was verified by restriction digestion and sequencing of the entire insert. Details will be provided on request.

Antigen expression and purification. The plasmids were transformed into the *Escherichia coli* expression strain BL21(DE3). The expression clones were cultivated in shaking flasks at 160 rpm and 37°C. Protein overexpression was induced by addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at an optical density at 600 nm (OD_{600}) of 0.6. After 4 h, cells were harvested by centrifugation at 5,000 rpm for 15 min (Sorvall RC6+; SLA3000; Thermo Scientific) and resuspended in buffer A (20 mM Tris, 0.5 M NaCl, 20 mM imidazole, pH 7.4). Cell disruption was achieved enzymatically by incubation of the cells with lysozyme (1 mg/ml) for 30 min on ice and by eight sonication steps with 1 min of cooling time between steps (cycle, 70%; power, 50%; Bandelin sonifier). The cell suspension was centrifuged at 15,000 rpm for 30 min to eliminate cell debris, and the supernatant was used for purification. Coomassie-stained SDS-PAGE and Western blotting confirmed the overexpression and solubility of the different recombinant proteins. The soluble histidine-tagged proteins were purified using affinity chromatography (HisTrap crude; GE Healthcare). As a second polishing step and for buffer exchange, size exclusion chromatography (Superdex 75; GE Healthcare) was performed. The relevant fractions were collected, concentrated if necessary with a centrifugal filter device (Millipore) with a cutoff of 10 kDa, and stored at −80°C. For insoluble proteins, 8 M urea was added to the lysis and purification buffers. In some cases, the protein was fused to a maltose binding protein to increase solubility. Details of purification are shown in Fig. S2 and S3 and Table S4 in the supplemental material.

Validation of antigens on the line assay *recom*Line. The newly developed test (*recom*Line) is a line immunoassay based on recombinantly expressed *H. pylori* proteins. In contrast to ELISA, the test principle allows the identification of specific antibodies against various antigens of *H. pylori* through the separate application of different single antigens. Highly purified recombinant *H. pylori* antigens were fixed on nitrocellulose membrane strips.

The appropriate line conditions for all recombinant antigens were determined empirically with a selection of standard serum samples from the study population described above. The optimal antigen concentration and ideal choice of additives, like detergent, dithiothreitol, and NaCl, was adjusted by repeated applications of each single antigen to the nitrocellulose membrane. The conditions with the best presentation of antigen epitopes and optimal binding to the membrane, characterized by consistent coloring, sharp bands, and the best discrimination of negative and positive samples, were chosen as the best production specifications of the first lots.

Production and processing of the line assay. For production of the newly developed *Helicobacter* line assay, individual dilutions of the purified recombinant antigens were applied directly onto nitrocellulose membranes in different lines using a BioDot XY (BioDot, England) front-line system and incubated under defined dry conditions for 2 h. The membranes were blocked with 1% skim milk solution in phosphate-buffered saline, air dried, and cut into individual test strips. The strips were stored at 4°C. Processing of the nitrocellulose test strips was performed as follows. Serum samples were applied to the nitrocellulose test strips at 1:100 dilutions in assay buffer (skim milk solution in Tris-buffered saline) and incubated for 1 h at room temperature. Titration experiments revealed that the 1:100 dilution displays the best signal-to-noise ratio combined with easy processing of samples. Following three washing steps of 5 min each in assay buffer, a second incubation of 45 min with peroxidase-labeled secondary antibody (anti-human IgG conjugate) was performed. The conjugate dilution of the kit component was 1:100 and was adjusted to overrun bound serum antibodies. After three additional 5-minute washing steps with assay buffer, the strips were stained for 8 min using tetramethylbenzidine. The coloring reaction was stopped with distilled water, and the strips were dried between thick layers of absorbent paper. For test analysis, as an alternative to visual reading, the scanner OpticPro S28 (Plustek, South Korea) and *recom*Scan software (Mikrogen, Germany) can be used according to the manufacturer's instructions. The test

was also analyzed manually by direct comparison of antigen reactivity with the cutoff band provided on every strip.

Endoscopy- and biopsy-based determination of *H. pylori* status. Endoscopy was performed on all patients after informed consent, and biopsy specimens from the antrum and corpus (two each) were taken from each patient. Histologic assessment was done on hematoxylin and eosin (H&E)-, Giemsa-, and Warthin-Starry-stained sections by two independent pathologists, and the samples were rated according to the updated Sydney System (40). Before endoscopy, 10 ml of venous blood was collected from each patient and centrifuged, and the sera were stored at −20°C.

ELISA. ELISA for anti-*H. pylori* IgG antibodies was performed using the commercially available test kit *recom*Well ELISA (Mikrogen, Germany) according to the instructions of the manufacturer. Plates for this ELISA are coated with recombinant CagA and GroEL. Serum samples are used at a 1:100 dilution and incubated with the antigen-coated plates for 1 h at 37°C. After three washing steps, a second incubation for 30 min with peroxidase-labeled secondary antibody (anti-IgG conjugate) was performed. An additional four washing steps were followed by a 30-min incubation in tetramethylbenzidine for color development. The optical density was measured with a commercial ELISA reader.

Immunoblot *Helicobacter*. The Immunoblot *Helicobacter* (Mikrogen, Germany) is a blot assay on which a whole-cell *H. pylori* lysate from a VacA- and CagA-positive *H. pylori* isolate is separated by gel electrophoresis and then blotted onto a nitrocellulose membrane. The membrane is then incubated with a protein solution to block free binding sites. As a reaction control, anti-human immunoglobulin, as well as positive and negative controls, is included. The test was carried out according to the instructions of the manufacturer.

Statistical analysis. Statistical analysis was done using IBM SPSS Statistics 20. The patients' characteristics are reported as means and standard deviations (SD) and absolute and relative frequencies. The diagnostic abilities of different serological tests were evaluated using sensitivity, specificity, and positive (PPV) and negative (NPV) predictive values. The PPV and NPV were calculated with a prevalence of 0.4. The values are given with 95% confidence intervals. Each serological test method was compared to histology as the gold standard. Sensitivity and specificity parameters of different serological assays were compared using the McNemar test.

RESULTS

Cloning, expression, and purification of antigens. Nine initially selected antigens were cloned into expression plasmids, resulting in pDS1_CagA, pQE30_VacA (p58), pUC8_GroEL, pDS1_UreA, pDS1_UreB, pMal_HcpC, pQE2_gGT, pQE2_Omp18, and pQE2_ICD. For the proteins CagA, GroEL, HcpC, gGT, Omp18, UreA, UreB, and ICD, the full-length sequence was used for amplification. In the case of VacA, only the midregion of the secreted fragment (p58) from a type m1 strain was used for cloning. For HcpC, an N-terminal maltose binding protein domain was introduced to increase solubility. The VacA protein and the two subunits from the urease (UreA and UreB) were expressed as inclusion bodies, and 8 M urea was added to the cell disruption and purification buffers for denaturation. The protein ICD, which also showed a band in the insoluble fraction, was denatured with 6 M guanidine hydrochloride and refolded by rapid dilution before purification. In the cases of CagA, GroEL, gGT, HcpC, and Omp18, SDS-PAGE showed a band in the supernatant, and these proteins could be used for purification without any further extraction steps. All selected proteins were purified to a concentration of ~1 mg/ml with a purity of >90% (see Table S4 in the supplemental material).

Selection of antigens. To investigate the immunogenicity of

TABLE 1 Characteristics of the 500 patients included in the study

Characteristic	Value		
	Histology		
	Negative	Positive	Total
Total no. (%)	290 (58.0)	210 (42.0)	500 (100)
Age (yr)			
Mean value	48.5	52.3	50.1
SD	16.0	16.2	16.2
Minimum	18.0	18.0	18.0
Maximum	86.0	86.0	86.0
Gender [no. (%)]			
Female	154 (53.1)	109 (51.9)	263 (52.6)
Male	136 (46.9)	101 (48.1)	237 (47.4)

individual antigens and to exclude cross-reactivity, validation testing was performed. All recombinant proteins were applied to a nitrocellulose membrane at different concentrations to determine the optimal concentration and buffer conditions for every individual antigen and were tested in repetitive rounds on a panel of blood donors whose *H. pylori* status was known from previous serological tests ($n = 42$) and patients whose *H. pylori* status had been verified by histology (number positive [n_{pos}] = 20; number negative [n_{neg}] = 15). The antigens CagA, VacA, GroEL, UreA, HcpC, and gGT showed clear immune responses with reasonable sensitivities and no or low cross-reactivity in negative samples (see Fig. S5 to S10 in the supplemental material). Also, these antigens showed the best differentiation between negative and positive patients. Therefore, the antigens were included in the test for further evaluation in a larger patient cohort. Other antigens, like UreB, Omp18, and ICD, showed cross-reactivity in noninfected patients at the concentrations used and were excluded from the test (see Fig. S11 in the supplemental material).

Patient population. A total of 500 patients were included in the study. The age distribution was between 18 and 86, with a mean age of 50.1 ± 16.2 years. The female-male distribution was 52.6% and 47.4%, respectively. Of the 500 patients tested, 290 (58.0%) were negative and 210 (42.0%) were positive for *H. pylori* in histology. The patients' characteristics are shown in Table 1.

Design of *recom*Line. The performance of serological *H. pylori* tests is highly dependent on the choice of antigens. The aim was to select antigens with a high prevalence in infected patients to increase the hit range of the assay. Furthermore, the antigens used should not cross-react with proteins of other species in order to increase the specificity. Moreover, they should be highly conserved in different *H. pylori* strains to be applicable in different patient populations. In order to be able to discriminate between *H. pylori* type I and type II strain infections, various virulence factors with known functions were selected. To achieve high reproducibility and robustness of the test, a line assay format was selected. Here, six recombinant proteins (CagA, VacA, GroEL, UreA, HcpC, and gGT) showing high immune responses in positive patients and no reactivity in negative patients were applied in separate lines to a nitrocellulose membrane to detect serological IgG immune responses against the antigens. Control bands were added on the upper ends of the strips comprising rabbit anti-human IgG/IgA antibodies as incubation controls, human IgG or IgA antibodies as conjugate controls, and a cutoff control that

				CagA	VacA	GroEL	UreA	HcpC	gGT	Σ
	IgG	IgA		2	2	2	1	1	1	
1			1	2						2
2			2			2	1	1	1	5
3			3					1	1	2
4			4					1		1
5			5	2	2	2	1	1	1	9

FIG 1 Total score points of patients showing different IgG immune responses. The antigens CagA, VacA, and GroEL score 2 points when positive. The antigens UreA, HcpC, and gGT score 1 point when positive. The total score (Σ) reaches 9 points when all antigen bands show up, as illustrated in strip 5. The test is considered positive with a score of 2 or more. If only one antigen with 1 score point shows a band, the result is judged to be borderline and the test should be repeated.

allows the assessment of the reactivities of the individual antigen bands. To allow discrimination between positive and negative patients, score points were assigned to individual antigens. CagA, VacA, and GroEL score 2 points because of their important clinical relevance, as documented in the literature (23). A positive UreA, HcpC, or gGT band scores 1 point. The test is considered positive with a score of 2 or more, and the maximum score is 9 points if all antigens are positive. If only a single antigen with 1 score point (UreA, HcpC, or gGT) is positive, the test is judged as borderline. This was the case for four patients showing a single band for HcpC. These patients were retested with the same result. For statistical analysis, the borderline results were treated as negative. Three of these patients were in fact negative, but one was found to be positive by histological analysis later. If no band appears, the patient is negative with zero score points (Fig. 1). This scoring system revealed optimal test performance in terms of maximal sensitivity and specificity compared to the histologic results in this population.

Since some *H. pylori* strains have been shown to lead to more severe clinical outcomes than others, antigens like VacA and CagA were included. These proteins are linked to the development of ulcers or premalignant changes and gastric cancer, respectively (24, 25, 26). Thus, the assay might be able to distinguish between a type I (more virulent) strain infection if CagA and/or VacA are positive and a type II strain infection if only one or more of the other antigens (GroEL, UreA, HcpC, and gGT) are positive (Fig. 2).

***recom*Line antigen recognition in *H. pylori*-positive patients.** Of the 210 patients positive for *H. pylori* by histology, 205 (97.6%) were *recom*Line positive, showing immune responses to the antigens included. As illustrated in Fig. 3, seroprevalence of antibodies against CagA and GroEL was most frequent, at 66.2% and 88.1%, respectively. For antigens HcpC and gGT, the frequency was moderate, at 47.1% and 51.9%. Antibody recognition in the case of UreA was rather low, at 28.1%, and only 21.4% for VacA. Usually, type I strains are considered to harbor CagA and the VacA s1m1 variant, which were incorporated on this line assay.

Due to the low recognition rate of VacA, the antigen CagA was considered the best marker to detect a type I strain infection. Our evaluation showed that 66.2% of the positive patients tested harbored a CagA-positive *H. pylori* strain. Further studies on the dis-

criminative power of our test to differentiate between a type I and a type II strain infection are currently ongoing.

Performances of different serological tests. To calculate the performances of the different serological tests, 500 patient sera were tested and evaluated with histology as the gold standard. The ELISA recognized 181/210 (86%) positive patients, with 29/210 (14%) false-negative results; 277/290 (95.5%) were recognized as negative, with 13/290 (4.5%) false-positive results. The lysate blot recognized 179/210 (85%) positive patients, with 31/210 (15%)
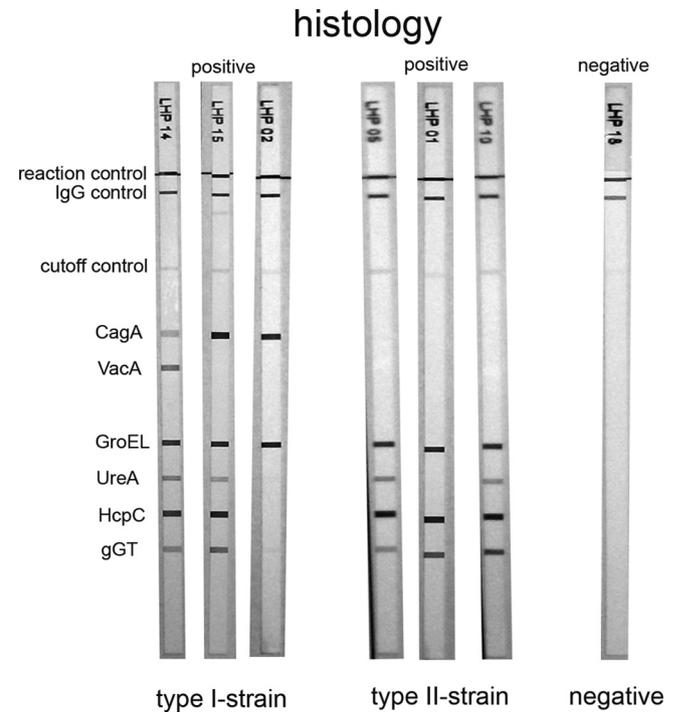

FIG 2 Results for detected immune responses of positive and negative sera verified by histology. The test is able to differentiate between type I and type II strain infections with the antigens CagA and VacA. In the top row, conjugate controls for detection of antibody (IgG) are included, serving as a positive control. To be able to discriminate the antigen bands from background staining, a cutoff control is included.
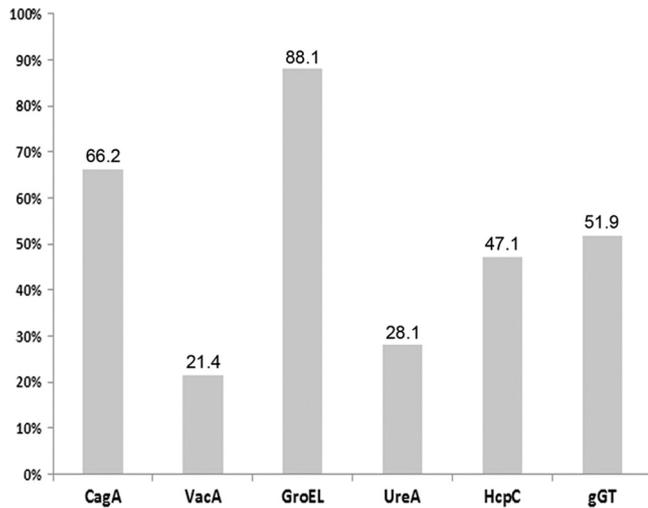
FIG 3 Antigen frequencies in the positive cohort. In total, 210 *H. pylori*-positive patients were tested. The antigens CagA and GroEL showed the highest recognition rates. Immune responses against HcpC and gGT were rather frequent, but the antigens VacA and UreA showed relatively low recognition rates.

TABLE 3 Comparison of different serological tests (*recom*Line, Immunoblot *Helicobacter*, and *recom*Well) to histology for detection of *H. pylori* infection[a]

Histology	*recom*Line	Immunoblot	*recom*Well	No./total
−	−	−	−	264/290
−	−	+	−	2/290
−	−	−	+	12/290
−	+	−	−	8/290
−	−	+	+	1/290
−	+	+	−	3/290
−	+	−	+	0/290
−	+	+	+	0/290
+	+	+	+	161/210
+	+	+	−	18/210
+	+	−	+	20/210
+	+	−	−	6/210
+	−	−	−	5/210
+	−	+	−	0/210
+	−	−	+	0/210
+	−	+	+	0/210

[a] −, negative; +, positive.

false-negative results. Of the 290 *H. pylori*-negative sera, 284 (98%) were negative, with 6/290 (2%) false-positive results. The *recom*Line assay recognized 205/210 (97.6%) positive patients, with 5/210 (2.4%) "false-negative" results (these five were negative in all serological tests applied); 279/290 (96.2%) were true negative, with 11/290 (3.8%) false-positive results. Referring to the patients' status as infected or noninfected, the sensitivity/specificity/PPV/NPV were 86.2/95.5/92.7/91.2 for the *recom*Well assay, 85.2/97.9/96.4/90.8 for the lysate blot, and 97.6/96.2/94.5/98.4 for the *recom*Line assay, respectively, as shown in Table 2. These data show that the sensitivity of the *recom*Line assay is significantly increased ($P < 0.001$; McNemar test) compared to both the *recom*Well and the lysate blot, with comparable specificity ($P = 0.227$ and $P = 0.839$, respectively).

Comparison of histology to serological *H. pylori* detection. To evaluate the new serological test against current state-of-the-art diagnostics, all patients in our population were examined by two independent experienced pathologists, and their clinical histories were assessed in most cases to exclude confounding factors, like prior eradication therapy or medication interfering with *H. pylori* colonization, such as proton pump inhibitors. A comparison of our serological results with the respective histologic examination is shown in Table 3.

Here, 264 (91%) of the 290 histologically negative patients were negative by all three serological tests. Two patients (0.7%)

showed a false-positive result only with the immunoblot assay, 12 (4.1%) with the *recom*Well assay, and 8 (2.8%) with the *recom*Line assay. In one case (0.3%), the lysate blot and the *recom*Well assay showed a false-positive result, and in three (1%) patients, the *recom*Line assay and the lysate blot gave false-positive results.

Of the positive patients, 161 (76.7%) of the 210 were true positive on all three tests. For 18 patients (8.6%), the *recom*Well ELISA showed false-negative results, and for 20 patients (9.5%), the immunoblot gave false-negative results. In six cases (2.9%), the immunoblot and the *recom*Well assay, and in five cases (2.4%) all serological tests, gave negative results while histology was positive.

DISCUSSION

Several tests are available for the detection of *H. pylori*. However, because of the high prevalence of infection, only a few tests are applicable for population-based screening approaches. First, the tests must be easy to handle and cheap to perform. Furthermore, they should have high sensitivity and specificity, because in a high-prevalence country like China, a false-positive rate of, e.g., 5% would lead to millions of patients receiving treatment without current infection. On the other hand, a false-negative rate of 5% would lead to high numbers of patients at risk for *H. pylori*-related diseases who would not receive treatment. Currently, no serological tests are available that meet all these requirements. Most tests rely on the use of single antigens, which do not confer sufficient sensitivity, or employ whole bacterial cell lysate, which leads to nonspecific reactivity and thus reduced specificity.

Serological assays based on recombinant *H. pylori* antigens are a promising alternative to tests based on whole-cell lysates. The line assay recombinant antigens in particular offer several major advantages: (i) inclusion of only specific antigens avoids confusion with comigrating nonspecific proteins, (ii) truncated antigens that contain only the specific or immunodominant part can be used, (iii) *in vivo*-produced antigens can be included, and (iv) standardization and evaluation are easier because the antigen compositions and concentrations can be adapted to requirements

TABLE 2 Performances of serological tests for the 500 cases included in the study

Parameter	Performance [% (no./total)]		
	*recom*Line	*recom*Well	Immunoblot
Sensitivity	97.6 (205/210)	86.2 (181/210)	85.2 (179/210)
Specificity	96.2 (279/290)	95.5 (277/290)	97.9 (284/290)
PPV	94.5	92.7	96.4
NPV	98.4	91.2	90.8

and therefore are not dependent on the actual expression pattern of the cultured strain.

The problem of low sensitivity of single antigens could be solved by combining different *H. pylori* antigens. The use of recombinant *H. pylori* antigens applied in separate lines onto a solid-phase test strip allows screening for diverse virulence factors from different *H. pylori* strains. Several pathogenic virulence factors are known, but they differ in immunogenicity and cross-reactivity.

The aim of the present study was to develop and evaluate a new line immunoassay based on recombinant *H. pylori* proteins in comparison to commonly used serological diagnostic methods, like tests based on *H. pylori* lysates. Histological examination was used as the gold standard. Therefore, we analyzed different antigens to evaluate the diagnostic value and the performance in routine diagnostics.

We were able to show that our new recombinant line immunoassay significantly increases sensitivity for IgG antibody detection compared to the IgG whole-cell lysate blot without a loss of specificity. Besides additional antigens, a major reason for this result is the fact that production of the line assay differs fundamentally from the classic whole-cell lysate immunoblot: for the line assay, each individual antigen, and even homologues, can be applied separately to the membrane at its optimized concentration and under independent buffer conditions. The antigens bind to the membrane without preceding SDS-PAGE or blotting. The line immunoassay is therefore cheaper and easier to standardize. The avoidance of SDS-PAGE or electrophoretic transfer to nitrocellulose membranes reduces the risk of epitope destruction. Small and high-molecular-weight antigens can be applied to the membrane under the same conditions, as there is no risk that small molecules may pass through the membrane driven by electrical force. Thus, the line assay system is independent of sources of errors related to gel electrophoresis, blotting, and the corresponding buffers. Furthermore, the line immunoassay offers the possibility of employing an automated analysis system, as every individual antigen is located at an exactly defined position on the strip.

The test we developed includes six antigens and shows high sensitivity and specificity (97.6% and 96.2%, respectively). It is worth mentioning that five patients who were "false negative" with the *recom*Line assay but positive by histology were also negative in all other serological tests employed. These included a whole-lysate immunoblot, which excludes the possibility that antigens other than those on the line blot might react. Rather, it is also possible that histology might be falsely positive in these patients, i.e., that bacteria other than *H. pylori* were detected in the stomach (despite the fact that Warthin-Starry staining was used). Alternatively, these patients might not mount a serologic response against the infection for various reasons, i.e., immunoglobulin deficiencies or immunosuppressive therapies, which might not have come to our attention.

Moreover, the test showed a PPV and NPV of 94.5% and 98.4%, respectively. Thus, we could improve the performance of the line immunoassay compared to the previous serological tests with respect to sensitivity and NPV while achieving comparable results for specificity and PPV. Reynders et al. (27) analyzed an immunoblot assay based on different recombinant antigens combined with native *H. pylori* antigens. They found the best antigen combination to be CagA, VacA, HP0175, p17, and p19. With this combination, the sensitivity, specificity, PPV, and NPV were reported to be 94%, 85%, 94%, and 95%, respectively (27). The low specificity could be due to the native antigens p17 and p19 that were employed, because they showed up to 20% nonspecific reactions in *H. pylori*-negative patients. Comparing these results with the data obtained with the *recom*Line assay illustrates the importance of careful selection of the antigens employed and the technical execution. For this reason, we performed several preevaluation and optimization steps, as exemplified in Fig. S5 to S11 in the supplemental material, in order to achieve the best signal-to-noise ratio.

A U.S. multicenter trial analyzed and compared three fingerstick whole-blood antibody tests for *H. pylori* infection. The study calculated sensitivities and specificities for these tests at 76 to 84% and 79 to 90%, respectively (28). Another study evaluated two serological tests with and without a marker for a presumed current infection. This immunoblot assay showed a sensitivity of 98%, comparable to that of the novel *recom*Line assay, but only a low specificity of 90% (29). This could be due to the fact that in this setting, whole *H. pylori* lysates were used, and some antigens included in the lysate might have given rise to false-positive results.

The antigen CagA is an important virulence factor indicating a type I *H. pylori* strain that harbors the *cag* pathogenicity island (*cag*-PAI). Infections with these strains correlate with the development of more severe gastroduodenal diseases, which makes CagA indispensable in serological tests. However, CagA is found in only a subgroup of *H. pylori* strains and thus is not suitable as a single marker for *H. pylori* infection. In our study, 34% (71/210) of the histologically positive patients did not show a CagA band; these patients would have been missed without employing other antigens. On the other hand, it is also reported that this protein can lead to anti-CagA reactivity in *H. pylori*-negative patients (30). In our study, only 1.7% (5/290) of the patients were "false positive" for CagA compared to 10% false-positive patients in the study of Fusconi et al. (30). Here, they considered this false-positive rate immunological memory due to a contact in the past, because there is no similarity to other bacterial or human peptides verified by alignment software. Therefore, CagA should be combined with other suitable antigens in serological tests to reliably confirm the seropositivity. In our population, *H. pylori*-positive patients recognized CagA in solely eight cases (3.8%). In total, the rate of CagA seropositivity was 66%, which represents the described prevalence of the *cag*-PAI in Western countries as shown by epidemiological studies (31).

VacA, which is involved in cellular cytotoxicity, as well as immune evasion (32, 33), had a rather low recognition rate in the *H. pylori*-positive patients. The protein comprises two different major regions: a signal sequence (s1a, s1b, or s2) and a midregion (m1 or m2), both with high allelic variation, which appears to influence cytotoxin activity (34). In particular, *vacA* s1m1 strains produce higher levels of cytotoxin activity than s1m2 strains, while s2m2 strains do not exhibit detectable cytotoxin activity. Infection with m1 strains may be associated with a high level of epithelial degeneration and erosion (35). This diversity, however, makes the detection of immune responses to VacA difficult in serologic settings, as not all forms can be detected. For our setting, we used the p58 fragment of an s1m1 strain that showed a low recognition rate in positive patients, which could be due to the high genetic diversity of the protein described above. Therefore, we might have missed patients with other variants of the protein. Reynders and

colleagues used the complete p95 fragment in their setting, which also showed only a low recognition rate of <20%, confirming this finding. To solve this problem, both forms of the midregion (m1 and m2) could be expressed and subjected to serologic detection. Furthermore, VacA had to be purified under denaturing conditions using 8 M urea, which might also affect the immunogenicity of the antigen.

Urease subunit A (UreA) also showed a rather low frequency of serological detection in the *H. pylori*-positive patients, but compared to subunit B (UreB), it showed reduced cross-reactivity. The low frequency of response to UreA could be due to the fact that the enzyme urease plays an important role at the time of colonization, but after the infection becomes chronic, urease levels may decrease. However, it has also been shown that the *H. pylori* urease makes up as much as 10% of the total cytoplasmic proteins (36), and therefore, it should be recognized rather well. Thus, our findings support previous findings that this protein might be a poor immunogen during natural infection *in vivo* (37).

gGT was recognized in half of the patients, but we noticed a stability problem on test strips stored for several months. Endogenous gGT consists of two subunits, one large subunit of 40 kDa and a smaller one of approximately 20 kDa, which are produced by autoprocessing of the 60-kDa precursor (38). This autocleavage could also occur on the test strip, which influences the detectability of the antigen. This could be addressed by expressing a mutant gGT lacking autoprocessing, which might help to increase stability. However, gGT has been described as an essential secreted virulence factor of *H. pylori* involved in immune evasion (39). It is therefore conceivable that a strong immune response against gGT might interfere with or be deleterious to successful colonization.

The two antigens GroEL and HcpC were recognized rather frequently and did not show cross-reactivity in *H. pylori*-negative patients. Also, it has been shown in different studies that these two antigens are found more often in patients with gastrointestinal disorders (23). These findings make GroEL and HcpC interesting for serological testing, as they show good immune responses in infected patients.

Compared to the other test systems in this study, the *recom*Line assay showed superior performance in regard to sensitivity and NPV and achieved comparable results for specificity and PPV. While histology is still considered the gold standard for *H. pylori*, it requires invasive measures and is not suitable for screening. Thus, a serological test system such as the one described here perfectly fulfills the need for a cost-effective but highly reliable test suitable for routine diagnostics and also for population-based approaches. Absolute costs, however, are difficult to calculate, as prices vary in different countries and between different manufacturers. The price for *recom*Line *Helicobacter* is, like the UBT, in a low one-digit Euro range but requires far less labor to be performed. In general, UBT shows similarly high performance, but in Germany, it is only recommended to determine treatment success (14). Therefore, the *recom*Line *Helicobacter* assay is the first assay that can be employed to reliably screen large populations. Moreover, it includes the option to identify infections by more pathogenic *H. pylori* strains, which might provide the basis for further treatment decisions. Further studies comparing the patient's immune responses with the genetic profile of the infecting *H. pylori* strain are ongoing and will be published elsewhere.

Taking the data together, our study shows that serological *H. pylori* detection with recombinant antigens enables us to detect infection with high sensitivity and specificity. As the line technique is more robust, it is easier to standardize and highly reliable. The recombinant line immunoassay therefore might be especially suitable for routine laboratories where large series of sera need to be investigated.

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