Comparison of Different Criteria for Interpretation of Immunoglobulin G Immunoblotting Results for Diagnosis of Helicobacter pylori Infection

Philipp M. Lepper,1,2* Angelika Möricke,1 Konstanze Vogt,3 Günter Bode,4 and Matthias Trautmann1†

Department of Medical Microbiology and Hygiene,1 Department of Epidemiology,2 and Second Department of Internal Medicine II,2 University of Ulm, Ulm, and Institute of Medical Microbiology, Charité, Humboldt University, Berlin,3 Germany

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Gastric infection with Helicobacter pylori is one of the most common chronic infections in humans, causing substantial morbidity and mortality (2). The diagnosis of H. pylori infection usually involves upper endoscopy with biopsy since the only noninvasive method of comparable accuracy, the [13C]urea breath test, requires technical equipment that is not available in most gastroenterological units. Serological methods for detection of H. pylori infection have reached sufficient accuracy to be used as screening tests before endoscopy or for seroepidemiological surveys. In the present study we evaluated different interpretation criteria for use with immunoglobulin G immunoblotting for the diagnosis of H. pylori infection. We applied five different sets of interpretation criteria, four of which had been published previously, to the western blot results of 294 patients with different gastrointestinal symptoms. Since it is known that less than 2% of patients who are infected with H. pylori fail to seroconvert, an optimally sensitive western blotting system should be able to detect approximately 98% of active infections. When the different criteria were applied to our patient population, it became apparent that the abilities of the systems to detect active H. pylori infection were quite varied. The results for the sensitivity and specificity, according to the different applied criteria, ranged from 62.8 to 95.9% and from 85.7 to 100.0%, respectively. Positive predictive values and negative predictive values, according to the published criteria, ranged from 97.2 to 100.0% and from 37.7 to 82.4%, respectively. Recommendations for the optimal use of the different interpretation criteria are discussed.

Gastric infection with Helicobacter pylori is one of the most common chronic infections in humans, causing substantial morbidity and mortality (2). The diagnosis of H. pylori infection usually involves upper endoscopy with biopsy since the only noninvasive method of comparable accuracy, the [13C]urea breath test, requires technical equipment that is not available in most gastroenterological departments (15). However, in recent years, serological methods for detection of H. pylori infection have reached sufficient accuracy to be used as screening tests before endoscopy or for seroepidemiological surveys (17, 26, 31–33, 36, 37).

Most of the commercially available enzyme-linked immunosorbent assay (ELISA) systems are based on the detection of immunoglobulin G (IgG) antibodies against whole-cell preparations of H. pylori (14, 16, 17, 23, 24). Such preparations include antigens cross-reacting with other bacterial species, resulting in a specificity of these tests that rarely exceeds 90% (36, 37, 38). Even with a second-generation ELISA system employing highly purified, presumably non-cross-reactive components of the outer membrane of H. pylori as antigens, we found a sensitivity of 97.2% but a specificity of no more than 87.5% in a population of 71 H. pylori-positive and 56 H. pylori-negative patients (37). However, if the decision to treat a patient for H. pylori infection is based solely on the result of serological tests as has been suggested recently (31, 35), there is a definite need for tests offering a higher specificity. Western blotting may be a useful tool in this respect because it allows the direct visualization of antibody binding to antigens highly specific for H. pylori (40). Furthermore, it is well known that the H. pylori phenotype possessing the virulence island of genes called the cytotoxin-associated gene complex (CagA) is associated with severe gastric pathology, such as ulcer disease and cancer (4). Western blotting may help to recognize infections caused by CagA-expressing H. pylori strains because the cytotoxin-associated protein is highly immunogenic and usually stimulates an IgG immune response against the corresponding antibody of 118 to 136 kDa (40).

In spite of these potential advantages and the widespread availability of blotting equipment in clinical laboratories, Western blotting for H. pylori antibodies has not become general practice. This is due mainly to discrepant recommendations for blot interpretation which have been published by various authors and that have resulted in considerable confusion regarding interpretation criteria. Comparative studies in which these different interpretation criteria have been compared using identical patient populations are not available. In the present study, we therefore used a collection of sera from patients known to harbor or not to harbor H. pylori in order to compare the levels of accuracy of the immunoblot diagnoses depending on different previously published and our own criteria.

* Corresponding author. Mailing address: Department of Internal Medicine II, University of Ulm, Robert-Koch-Str. 8, 89081 Ulm, Germany. Phone: 49 731 500 40393. Fax: 49 731 500 24521. E-mail: philipp.lepper@medizin.uni-ulm.de.
† Present address: Katharinenhospital, Institute for Hospital Hygiene, Kriegsbergstr. 60, 70174 Stuttgart, Germany.
TABLE 1. Summary of patient data

| Group | No. of patients | H. pylori status | Endoscopic and histopathologic diagnosis | Mean age (yr) ± 1 SD | No. of males (% of group) |
|-------|----------------|-----------------|-----------------------------------------|----------------------|-------------------------|
| 1     | 57             | +               | Duodenal ulcer                          | 56.5 ± 16.9          | 37 (64.9)               |
| 2     | 42             | +               | Gastric ulcer                           | 57.8 ± 15.0          | 15 (55.7)               |
| 3     | 38             | +               | Gastritis                               | 59.6 ± 17.1          | 20 (52.6)               |
| 4     | 47             | +               | Normal                                  | 49.0 ± 17.9          | 23 (48.9)               |
| 5     | 83             | −               | Normal                                  | 50.7 ± 16.4          | 40 (48.2)               |
| 6     | 9              |                 | Normal                                  | 57.3 ± 13.2          | 3 (33.3)                |
| 7     | 6              | 5+, 1−          | Gastric MALToma                         | 53.3 ± 14.3          | 4 (66.7)                |
| 8     | 12             | 10+, 2−         | Gastric carcinoma                       | NA                   | NA                      |
| Σ     | 294            |                 |                                         | 54.5 ± 17.5          | 142 (50.3)              |

* NA, not available.

RESULTS

Patients. Forty patients had to be excluded from further analysis because of insufficient or unreliable information concerning their gastric H. pylori status (most often because the result of the histopathological examination was insufficient for a diagnosis of H. pylori infection).

A summary of the remaining 294 patients is given in Table 1. H. pylori infection was detected in 47 asymptomatic patients without gastric pathology, and 83 persons had no gastric abnormality and were H. pylori negative.

Western blot reaction patterns of patients. Figure 1 shows the band patterns of two strongly positive sera to illustrate the antigens present in the preparation and to demonstrate the variability of the IgG immune response. The CagA protein, which has been described by other authors as running with apparent molecular sizes between 110 and 138 kDa, was found to have a molecular size of 136 kDa in the present study. The 87-kDa band represents the VacA protein, the 66-kDa band represents the urease B subunit, and the 59- and 55-kDa proteins are thought to be components of the H. pylori flagella.
The double-band staining of the 66-kDa (UreaB) band seen in lane 1 of Fig. 1 is typical for this band and has also been described when blots were reacted with a monoclonal antibody raised against this protein (18). The 59-kDa band may also be related to the 60-kDa, urease-associated heat shock protein identified in \textit{H. pylori} (11), or the two bands may actually run so close to each other that they cannot be separated under the electrophoretic conditions used. It should, however, be noted that the heat shock protein has also been described as having a molecular size of 54 kDa (9), and therefore, it may also be located within the 54-kDa band present in our preparation. The 30- and 19.5-kDa proteins have been described as species-specific proteins of \textit{H. pylori}, although their functional role is unclear at present (3, 7). The 26-kDa band represents the A subunit of the urease which has been described as running with apparent molecular sizes of 26 to 31 kDa (8, 19, 30).

Both of the two sera shown in Fig. 1 contained IgG antibodies binding to the CagA and VacA bands, although the VacA reaction was very faint in the serum shown in lane 2. The strongest reaction of the serum shown in lane 1 was directed against the 59-kDa probe. No antibody against the 30-kDa species-specific protein was present. By contrast, the serum represented in lane 2 showed the strongest reactions with the urease B subunit (66 kDa) and the 30-kDa protein. Both sera recognized the 26- and 24-kDa proteins, although some variations of the apparent molecular size, shown with double banding in lane 2, were obvious. This phenomenon, which has also been observed by others (30), may be explained by the different epitope specificities of the antibodies directed against individual surface proteins, which actually occupy a relatively broad space on the blot strip.

Typical band patterns produced by other sera from \textit{H. pylori}-positive and -negative patients are shown in Fig. 2. Prominent bands seen in the majority of positive sera were the 136-, 74-, 66-, 59-, 55-, 50-, 26-, and 24-kDa bands. Again, the variability of the reaction with the 26- and 24-kDa bands was obvious, although these sera were run on blot strips produced from the same electrophoretic run. The 87-kDa (VacA) band stained weakly in blots of sera from some patients with active \textit{H. pylori} infection (Fig. 2A and B) and one successfully treated patient (Fig. 2D) but was not found in blots for asymptomatic \textit{H. pylori}-negative patients (Fig. 2C). The 136-kDa (CagA) band was seen in blots of sera from most of the patients with invasive \textit{H. pylori} infection; however, it was clearly absent from some of them (Fig. 2B, lanes 2, 4, 5, and 7). It is faintly visible in lanes 9 and 10 of the original blots. Conversely, this band, although faintly stained, was also detectable on the original blots for a significant proportion of asymptomatic, \textit{H. pylori}-negative patients (Fig. 2C, lanes 5, 7, 8, 9, 11, and 13).

The intensity of band staining was clearly correlated with disease activity (Table 2). The mean OD values of positive sera were strongest for the 59-kDa band, followed by the 55-, 136-, and 50-kDa bands. Sera from patients who did not at that time harbor \textit{H. pylori} showed positive reactions most often with the 55- and 59-kDa (flagellin complex) probes (Table 2). Seventeen (20.5%) sera of \textit{H. pylori}-negative persons without gastric disease clearly reacted with the 136-kDa (CagA) probe.

Table 3 summarizes the calculated seropositivity rates according to the different interpretation criteria. Figure 3 shows the graphic representation of these rates. The lowest seropositivity rate in the group of \textit{H. pylori}-negative patients (group 5) was obtained with the criteria of von Wulffen et al., while the highest rate was reached with the criteria that we propose in this study.

**DISCUSSION**

Although a number of authors have studied the humoral immune response against \textit{H. pylori} by Western immunoblotting, most of the publications dealing with this subject are merely descriptive (1, 5, 12, 19, 23, 24, 38). We found only four studies (13, 30, 37, 39) in which interpretation criteria were proposed. In the present study, we evaluated the performance of blotting according to these criteria.

One of the interpretation systems used a quantitative cutoff value for band intensity (39), while the others considered bands of any intensity. Furthermore, bands representing the flagellar complex (59 and 55 kDa) were to be included in the analysis, although these proteins have been shown to be immunologically cross-reactive among flagellated bacteria of var-
FIG. 2. Examples of the immunoblot reaction patterns of representative sera from *H. pylori*-positive and *H. pylori*-negative patients. Markers to the left of each panel are in kilodaltons. (A) Patients from group 1 (*H. pylori* positive with duodenal ulcer [n = 57]). (B) Patients from group 2 (*H. pylori* positive with gastric ulcer [n = 42]). The 136-kDa band is absent from lanes 2, 4, 5, and 7; it is, however, faintly visible (on the original blots) in lanes 9 and 10. (C) Patients from group 5 (*H. pylori* negative with no gastric disease [n = 83]). The 136-kDa band is absent from lanes 1, 2, 3, 4, 6, 10, 12, 13, and 14 and faintly visible in lanes 5, 7, 8, 9, and 11. (D) Patients from group 6 (currently *H. pylori* negative with no gastric disease, previously underwent *H. pylori* eradication therapy [n = 9]). (A, B, and D) Arrowheads indicate the 87-kDa (VacA) band.
negative with no gastric disease. HS, highly specific.

It became apparent that the abilities of the systems to detect positive bands of these molecular sizes. The other interpretation systems do not consider these bands (30, 37, 39).

In applying the different criteria to our patient population, it became apparent that the abilities of the systems to detect active *H. pylori* infection were quite varied. Since it is known that less than 2% of patients who harbor *H. pylori* fail to seroconvert (17, 20, 21, 34, 35, 37), an optimally sensitive Western blotting system should be able to detect approximately 98% of active infections. The only interpretation system that approached this sensitivity was our currently used system (Tables 3 and 4). The main difference between our current interpretation criteria and those proposed by Nilsson et al. (30) is that those authors considered a 94-kDa band in their analysis instead of the 66-kDa (urease B) band included in our system.

The 94-kDa band described by Nilsson et al. most probably corresponded to the urease B subunit of *H. pylori* because they did not react with strains belonging to other urease-producing species and genera, such as *Clostridium perfringens*, *Enterobacter*, *Pseudomonas*, and, most notably, *Campylobacter* (18). A similar mono-

| Molecular size (kDa) of antigen | Specificity | Groups 1 & 2 (n = 99) | Group 4 (n = 47) | Group 5 (n = 83) |
|-------------------------------|------------|------------------------|------------------|------------------|
| 136 (CagA)                    | HS         | 75 (75.8) 0.48 ± 0.42  | 24 (51.1) 0.49 ± 0.45 | 17 (20.5) 0.18 ± 0.19 |
| 87 (VacA)                     | HS         | 53 (35.4) 0.10 ± 0.08  | 11 (23.4) 0.06 ± 0.06 | 2 (2.4) 0.07 ± 0.03 |
| 66 (UreB)                     | NS         | 66 (66.7) 0.34 ± 0.45  | 26 (55.3) 0.53 ± 0.74 | 26 (31.3) 0.14 ± 0.27 |
| 59 (HspB)                     | Specific    | 81 (81.8) 1.45 ± 1.26  | 32 (68.1) 1.62 ± 1.33 | 30 (36.1) 0.19 ± 0.39 |
| 55 (FlaA/FilaB)               | Specific    | 88 (88.9) 0.54 ± 0.61  | 41 (87.2) 0.65 ± 0.81 | 49 (59.0) 0.15 ± 0.23 |
| 50                            | Specific    | 65 (65.7) 0.40 ± 0.56  | 27 (57.4) 0.45 ± 0.62 | 10 (12.0) 0.26 ± 0.45 |
| 45                            | Specific    | 26 (26.3) 0.20 ± 0.20  | 18 (38.3) 0.27 ± 0.42 | 8 (9.6) 0.11 ± 0.14 |
| 33                            | HS         | 10 (10.1) 0.26 ± 0.40  | 4 (8.5) 0.05 ± 0.03 | 10 (12.0) 0.06 ± 0.06 |
| 30                            | HS         | 20 (20.2) 0.29 ± 0.24  | 12 (25.5) 0.35 ± 0.62 | 9 (10.8) 0.06 ± 0.03 |
| 26 (UreA)                     | HS         | 53 (53.5) 0.33 ± 0.48  | 20 (42.6) 0.26 ± 0.27 | 24 (28.9) 0.10 ± 0.13 |
| 24                            | HS         | 57 (57.6) 0.33 ± 0.44  | 27 (57.4) 0.40 ± 0.69 | 26 (31.3) 0.07 ± 0.06 |
| 19.5                          | HS         | 53 (53.5) 0.33 ± 0.44  | NA               | 14 (16.9) NA       |

* Group 1, *H. pylori* positive with duodenal ulcer; group 2, *H. pylori* positive with gastric ulcer; group 4, *H. pylori* positive with no gastric disease; group 5, *H. pylori* negative with no gastric disease. HS, highly specific for *H. pylori*; NS, nonspecific for *H. pylori*; NA, not available.

| Molecular size (kDa) of antigen | Specificity | Groups 1 & 2 (n = 99) | Group 4 (n = 47) | Group 5 (n = 83) |
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| 19.5                          | HS         | 53 (53.5) 0.33 ± 0.44  | NA               | 14 (16.9) NA       |
H. pylori-negative with no gastric disease, previously underwent H. pylori ELISA system to detect clonal antibody has been used to construct a competitive ELISA system to detect H. pylori-specific IgG antibody in the sera of patients. This system was both highly sensitive and specific for the detection of H. pylori infection (28).

In order to evaluate the specificity of blotting, it would be necessary to know the number of truly H. pylori-seronegative persons. From the data shown in Table 3 it is clearly evident that this value cannot be identical with the number of patients without current H. pylori infection (group 5), because all interpretation systems recognized some patients of this group as seropositive. Since we took at least three biopsies per patient and examined them by three different methods, each having a sensitivity of >90% to detect H. pylori (6), we do not believe that sampling errors of the biopsy technique can explain the mismatch between the results of reference methods and serology. Rather, it must be assumed that an unknown percentage of patients in this group had residual IgG antibodies after former H. pylori infection. While spontaneous elimination of H. pylori may occur more frequently than is usually assumed (16), persons included in this group may also have been incidentally cured during antibiotic treatment of respiratory or other infections (34). In this respect, it is of interest that Meyer et al. saw the same mismatch between the prevalence of H. pylori antibodies in serum and [13C]urea breath test results (27); among 100 healthy volunteers, 49 had H. pylori antibodies, whereas only 24 had a positive breath test as an indicator of active infection (27).

In order to estimate the true number of H. pylori-seropositive persons in our population (group 5), the following assumptions were made: it has been shown that approximately 50% of healthy, H. pylori-seropositive persons from industrialized countries have antibody against CagA in their sera (32, 33). This figure was again confirmed in our study with the patients of group 4, in which a 51% prevalence of CagA antibodies was found (Table 2). Since 17 patients (20.5%) in group 5 had antibodies against the CagA protein (e.g., Fig. 2C, lanes 5, 7 to 9, 11, and 13), and since we believe that this finding was unequivocal due to the clear delineation of the corresponding 136-kDa band, the overall number of H. pylori-seropositive persons in this group can be assumed to be twice as high, namely, ~34. Using 49 as a denominator and assuming that all positive Western blot results belonged to this seropositive group, the performance figures for the specificity given in Table 4 were calculated. In order to calculate the sensitivity, we

![FIG. 3. Overall blot positivity of eight diagnosis groups of H. pylori-positive patients according to different interpretation criteria. Group 1, H. pylori positive with duodenal ulcer (n = 57); group 2, H. pylori positive with gastric ulcer (n = 42); group 3, H. pylori positive with gastritis (n = 38); group 4, H. pylori positive with no gastric disease (n = 47); group 5, H. pylori negative with no gastric disease (n = 83); group 6, currently H. pylori negative with no gastric disease, previously underwent H. pylori eradication therapy (n = 9); group 7, 5 H. pylori-positive patients with gastric mucosa-associated lymphoid tissue lymphoma (MALToma) (n = 6); group 8, 10 H. pylori-positive patients with gastric carcinoma (n = 12). Only H. pylori-positive patients were considered for this figure.](image)

**TABLE 4. Statistical performance of H. pylori IgG immunoblotting according to different interpretation criteria**

| Authors of interpretation criteria | Sensitivity | Specificity | Positive predictive value | Negative predictive value |
|-----------------------------------|-------------|-------------|---------------------------|---------------------------|
| von Wulffen et al. (39)           | 137/218 (62.8) | 49/49 (100) | 152/152 (100) | 49/130 (37.7) |
| Faulde et al. (13)                | 152/218 (69.7) | 49/49 (100) | 165/165 (100) | 49/115 (42.6) |
| Trautmann et al. (37)            | 196/218 (89.9) | 49/49 (100) | 230/230 (100) | 49/71 (69.0) |
| Nilsson et al. (30)              | 183/218 (83.9) | 49/49 (100) | 214/214 (100) | 49/84 (59.3) |
| Lepper et al. (this study)       | 209/218 (95.9) | 42/49 (85.7) | 243/250 (97.2) | 42/51 (82.4) |

*Only patients from groups 1 to 5 (n = 267) were included in these calculations. The number of truly seropositive patients in group 5 (n = 83) was assumed to be 34 (41%; see Discussion), and the number of truly seronegative patients in group 5 was assumed to be 49.*
added the 34 patients assumed to be seropositive to the number of truly positive patients with either a positive or a negative Western blot result. Since the tests used to verify the \textit{H. pylori} status can detect only active infections, in contrast to Western blotting, and the possibility of a sampling error cannot be excluded for every patient, we believe that the addition of the seropositive patients of this group avoids the overestimation of the sensitivity. The resulting 218 patients were used as a denominator to calculate the sensitivity values shown in Table 4.

These data show that the interpretation systems of Wulffen et al. and Faulde et al. had low sensitivities and low negative predictive values. However, these systems and the system of Nilsson et al. yielded results that were 100% specific, i.e., the absence of (former and present) \textit{H. pylori} infection can be detected with 100% probability on the basis of these criteria. The interpretation system that combined a relatively high sensitivity (89.9%) with 100% specificity was that of Trautmann et al. (37). The positive predictive values of all of the systems were acceptable, but the negative predictive values were relatively low except with our newly developed criteria (Table 4). This means that a negative test result obtained with these systems does not reliably exclude the possibility of the presence of past or current \textit{H. pylori} infection.

Which of these criteria should be applied when performing \textit{H. pylori} Western blots? The answer to this question may depend on the purpose of the serological examination. If serology is to be used for seroepidemiological surveys, then Western blotting is a very useful tool because it detects both former and current \textit{H. pylori} infections. A system that combines the highest possible positive and negative predictive values may be most suitable for this purpose, and therefore, our newly developed criteria can be recommended for this application. If serology is to be used as a preendoscopic screening method, then the sensitivity must be very high (in order not to miss a patient who might harbor \textit{H. pylori}), but the specificity is of lesser concern because false-positive serological findings will be corrected by the results of the invasive tests that are going to follow. Our presently proposed criteria are recommended for this purpose. If the results of serological tests are used to decide about \textit{H. pylori}-targeted therapy in patients with dyspepsia without performing endoscopy as has been recently suggested (31, 35), then the specificity and the positive predictive value must be as high as possible in order to avoid the unnecessary and potentially harmful treatment of patients who are not actually infected. In this case, the criteria of Nilsson et al. and Trautmann et al. may be most suitable. An additional advantage of Western blotting in this scenario is its ability to detect infection with CagA-positive \textit{H. pylori} strains, because treatment can be limited to the subgroup of CagA-seropositive patients (27).

Finally, it has been suggested that Western blotting may be used as a second-line method in a two-step serological workup (30). The first step is an ELISA which identifies all truly and potentially \textit{H. pylori}-positive patients with a high sensitivity. If serology is to be used for deciding about treatment, the ELISA method can be adjusted in such a way that it detects only currently \textit{H. pylori}-infected patients. Western blotting is then used as a second step in order to identify false-positive ELISA results. In this case, the sensitivity of blotting is of no primary importance, but a high specificity (i.e., ability to detect truly negative patients) is desired. The criteria of Nilsson et al. and Trautmann et al. would be most suitable for this purpose.

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