

# Sensitivity of *Helicobacter pylori* detection by Giemsa staining is poor in comparison with immunohistochemistry and fluorescent in situ hybridization and strongly depends on inflammatory activity

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## Abstract

**Background:** Conventional stainings (including H&E and special stains like Giemsa) are the most widely applied histopathologic detection methods of *Helicobacter pylori* (HP).

**Materials and Methods:** We aimed to compare the diagnostic performance of Giemsa staining with immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH) on a monocentric cohort of 2896 gastric biopsies and relate results to histologic alterations in order to find such histopathologic subgroups in which these methods underperform. All cases were categorized regarding presence or absence of chronic gastritis, inflammatory activity, and mucosal structural alterations.

**Results:** Giemsa revealed 687 cases (23.7%), IHC 795 cases (27.5%), and FISH 788 cases (27.2%) as being HP positive. Giemsa showed significantly lower overall sensitivity (83.3%) compared to IHC (98.8%) and FISH (98.0%). Moreover, the sensitivity of Giemsa dramatically dropped to 33.6% in the nonactive cases. We found that sensitivity of Giemsa strongly depends on HP density and, accordingly, on the presence of activity. Structural alterations (intestinal metaplasia, atrophy, etc.) had only no or weak effect on sensitivity of the three stainings. Both IHC and FISH proved to be equally reliable HP detecting techniques whose diagnostic performance is minimally influenced by mucosal inflammatory and structural alterations contrary to conventional stainings.

**Conclusions:** We highly recommend immunohistochemistry for clinically susceptible, nonactive chronic gastritis cases, if the conventional stain-based HP detection is negative. Moreover, we recommend to use IHC more widely as basic HP stain. *Helicobacter pylori* FISH technique is primarily recommended to determine bacterial clarithromycin resistance. Furthermore, it is another accurate diagnostic tool for HP.

## KEYWORDS

chronic gastritis, diagnostic algorithm, FISH, *Helicobacter pylori*, immunohistochemistry, sensitivity

## 1 | INTRODUCTION

*Helicobacter pylori* is a Gram-negative spiral-shaped bacterium, which colonizes the human stomach<sup>1-3</sup>, leading to chronic mucosal inflammation and thus playing a pivotal role in the pathogenesis of chronic gastritis-related diseases (peptic ulcer diseases, gastric adenocarcinoma, and mucosa-associated lymphoid tissue [MALT] lymphoma).<sup>4</sup>

Certain *H. pylori*-related gastric mucosa alterations have emphasized significance in setting up the histologic diagnosis. Neutrophil infiltration of the epithelium characteristically occurs and indicates an active mucosal inflammation known as the activity of chronic gastritis. Another notable feature of *H. pylori*-sustained chronic inflammation is the presence of structural alterations in the mucosa (eg., intestinal metaplasia, atrophy, erosion/ulceration).<sup>5</sup>

In clinical routine diagnostics, *H. pylori* can be detected via invasive (biopsy based) or noninvasive (eg., rapid urease test and serologic tests) procedures. The biopsy-based histopathologic examination is accepted as one of the gold standard methods to diagnose *H. pylori*.<sup>6,7</sup> For this purpose, conventional histologic stainings, including the basic histology staining hematoxylin and eosin (H&E) as well as special stainings, such as modified Giemsa (hereafter briefly: Giemsa)<sup>8</sup>, methylene-blue, and Warthin-Starry silver staining, are commonly used.<sup>7,9,10</sup> In addition, immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH)-based staining methods are also available.<sup>6,7,9,10</sup> Polymerase chain reaction (PCR)-based molecular techniques were also adapted to *H. pylori* diagnostics, however, with limitations, regarding an extreme sensitivity to contamination, the presence of cross-reacting nucleotide sequences and the high genetic variability of the bacterial strains.<sup>11-13</sup>

The primary aim of our study was to compare the sensitivity and specificity of the widely available Giemsa staining with IHC and FISH on a large monocentric cohort of gastric biopsies obtained for routine diagnostics. In addition, as a secondary goal, we wished to reveal whether the *H. pylori*-related changes of gastric mucosa could influence diagnostic performance of Giemsa, IHC, and FISH stainings in order to find such histopathologic subgroups in which one or more of these methods underperform.

## 2 | METHODS

### 2.1 | Patients, biopsies, and histologic work-up

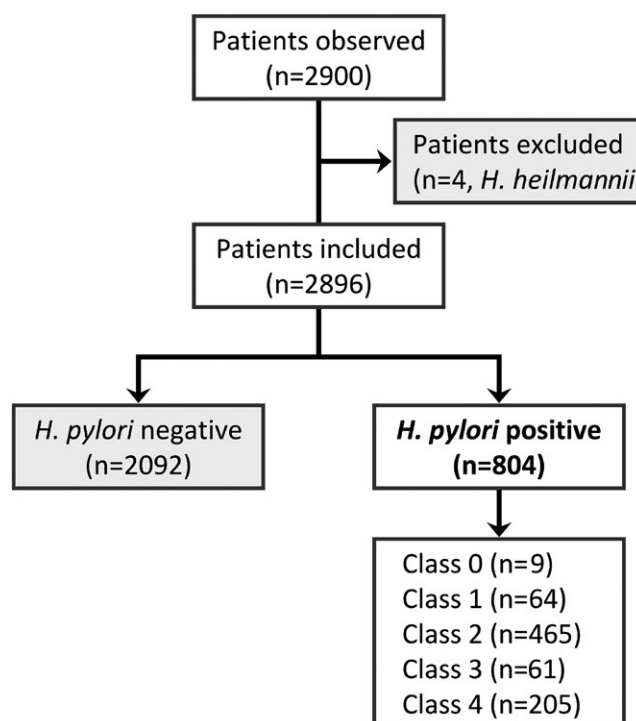
A total of 2900 patients (1200 males, 1700 females, mean age: 50.47 [SD:  $\pm 20.52$ ] years) were primarily included in the study, who underwent gastric endoscopy and biopsy due to upper abdominal complaints between 2006 and 2008. The study protocol was in accordance with the ethics guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Semmelweis University (#148/2007). Gastric biopsies were taken from the antral (n=3) and oxyntic (n=2) compartment mucosa, directly fixed in 10% buffered formalin, embedded in paraffin, cut into 3-4  $\mu$ m sections and stained with H&E, Giemsa, and Alcian Blue-Periodic acid-Schiff in accordance with our routine protocols. Gastritis was diagnosed and grouped according to the updated Sydney system.<sup>5</sup>

For statistical purposes, all cases were categorized with regard to the presence or absence of (1) chronic gastritis, (2) inflammatory activity, and (3) *H. pylori* and/or chronic gastritis-related structural alterations of the gastric mucosa (Table S1, Figure 1). The considered structural alterations included intestinal metaplasia, atrophy/preatrophy, erosion, ulcer, foveolar hyperplasia, hyperplastic polyp, dysplasia, carcinoma, lymphoid follicle, and MALT lymphoma. In short, the different statistical classes are as follows:

- 0: no chronic gastritis,
- 1: chronic gastritis without activity or structural alteration,
- 2: chronic gastritis with activity but without structural alteration,
- 3: chronic gastritis without activity but with structural alteration, and
- 4: chronic gastritis with both activity and structural alteration.

### 2.2 | Immunohistochemistry—staining procedures and status

Slide preparation was made separately for immunohistochemistry in a second sectioning process. Three freshly cut 4  $\mu$ m sections per biopsy specimen were mounted on a coated glass slide, deparaffinized, and microwaved (160 W, 30 minutes in Tris-EDTA buffer, pH 9.0) for antigen retrieval. B0471 polyclonal rabbit anti-*Helicobacter pylori* primary antibody (Dako, Glostrup, Denmark) was applied to the tissue in 1:350 dilution and incubated (32 minutes, 42°C) in Ventana 320 automated immunohistochemistry staining system, subsequently detected using iView DAB Detection Kit (Ventana Medical Systems, Inc. Tucson, AZ, USA). Hematoxylin was used as nuclear counterstain. Severity of the



**FIGURE 1** Flowchart of the case selection

*H. pylori* infections was graded semiquantitatively into mild (1+; low bacterial density, scattered *H. pylori* distribution pattern), moderate (2+; moderate bacterial density, small groups of *H. pylori*), or severe (3+; high bacterial density, large groups/fields of *H. pylori*) categories.

## 2.3 | Fluorescence in situ hybridization—staining procedures and status

Slide preparation was made for FISH during a separate, third sectioning. One freshly cut 4 µm section from each gastric mucosa containing biopsy specimen was mounted on a coated glass slide, deparaffinized, and microwaved at 400 W for 10 minutes in Vector Antigen Unmasking Solution H-3300 (Vector Laboratories, Burlingame, CA, USA). BACTFish *H. pylori* Combi Kit (Izinta Kft., Budapest, Hungary) was used to perform FISH on the studied samples. Prepared DNA hybridization solution containing DNA Probe Mix was added to the specimens and incubated for 90 minutes at 46°C. The DNA Probe Mix contained specific probes for the identification of *H. pylori* (16S-rRNS, labeled with green fluorochrome, FITC/fluorescein-isothiocyanate/), and for the detection of clarithromycin resistance (23S-rRNS, labeled with orange-red fluorescent Cy3). After posthybridization wash (15 minutes at 46°C), the slides were covered with mounting medium containing DAPI (4',6'-diamidino-2-phenylindole) fluorescent nuclear counterstain (Vector Laboratories, Burlingame, CA, USA). Specific signals were examined with epifluorescence microscope Leica DM-RXA (Leica, Wetzlar, Germany) equipped with Spectrum Green and Spectrum Orange filters (Vysis, Downers Grove, IL, USA) for green and orange-red fluorescence, respectively. Images were taken using Pieper FK-7512-IQ (Pieper GmbH, Schwerte, Germany) camera and documented by Leica CW4000 FISH analysis software. The presence of *H. pylori* was graded semiquantitatively into mild (1+), moderate (2+), and high (3+) categories for each examined tissue block as described above.

## 2.4 | *Helicobacter pylori* FISH on the originally Giemsa-stained slides (FISH on Giemsa)

In cases with discrepant staining results, an additional *H. pylori* FISH was performed on the originally Giemsa-stained slides (FISH on Giemsa), to verify the result of Giemsa staining by a more advanced technique. In these cases, the coverslip of the Giemsa-stained slide was removed, and then, to prevent the section from floating off slide, the uncovered tissue sample was tightly fixed onto the glass surface by coating with a fine plastic mesh (Flow-Mesh, cat. no. Z377619, Sigma-Aldrich, St. Louis, MO, USA). Afterward, FISH staining was carried out as described above. *H. pylori* FISH-negative cases with positive FISH on Giemsa were finally considered as FISH positive.

## 2.5 | *Helicobacter heilmannii*

*Helicobacter heilmannii*-positive cases were excluded from the statistical analyses. For the diagnosis of *Helicobacter heilmannii*, the following parameters had to be fulfilled: (1) special morphological appearance

(longer, more tightly coiled spiral bacterium than the *H. pylori*), (2) positive IHC reaction for *Helicobacter* antigen, and (3) negative *H. pylori* FISH.

## 2.6 | Statistical analyses

The primary aim of our study was to calculate the sensitivity, specificity, positive, and negative predictive values for each studied stain. As for our secondary goal, categorical data were compared using chi-square and (if chi-square test was insufficient to generate valid results) Fisher's exact probability tests. Holm-Bonferroni test was used for multiple comparisons. McNemar's test was applied for pairwise comparison of the Giemsa, IHC, and FISH staining techniques in the same population, subgroup, or statistical class. To compare results of the same staining method between the different statistical classes, each combination of classes was pair-matched and their results compared against each other using chi-square and/or Fisher's exact probability tests. Subsequently, the obtained P values were rank-ordered and their significance was examined by the Holm-Bonferroni method. Classification and regression tree (CART) analysis was applied to reveal the optimal diagnostic decision rules. All P values were calculated using two-tailed statistical tests, and differences were considered significant in general when  $P < .05$ , except for the Holm-Bonferroni method where the more accurate  $P < .01$  condition was used. CART was performed using the rpart package of R software ([www.r-project.org](http://www.r-project.org)). Other statistical analyses were performed using Statistica 12.0 (StatSoft Inc., Tulsa, OK, USA) and SPSS Statistics for Windows ver. 23 (IBM Corporation, Armonk, NY, USA) softwares.

## 3 | RESULTS

From our single institution cohort comprising 2900 patients, all of the gastric tissue specimens were stained with Giemsa, *Helicobacter* IHC, and *H. pylori* FISH. *H. heilmannii*-positive cases were excluded from the further analysis (none of these four cases were coinfecting with *H. pylori*) (Figure 1). Accordingly, evaluation of the three different stainings from the remaining 2896 patients revealed 687 cases (23.7%) to be positive for *H. pylori* with Giemsa, 795 cases (27.5%) with IHC, and 788 cases (27.2%) with FISH (Table 1 and Figure 2A-D).

### 3.1 | Sensitivity, specificity, positive, and negative predictive values

Based on the publication of Rotimi et al.,<sup>6</sup> immunohistochemistry was considered as a reference for diagnostic performance calculations of *H. pylori* stainings. Accordingly, Giemsa revealed an overall sensitivity of 83.3% and a specificity of 98.8%. Positive and negative *H. pylori* predictive values were 96.4% and 94.0%, respectively. Sensitivity of *H. pylori* FISH was 98% and specificity reached 99.6%. Positive and negative *H. pylori* predictive values of the FISH technique were 98.9% and 99.2%, respectively. Hence, FISH was used as a reference for the reverse comparison, in which sensitivity of IHC was 98.8% and

|            | Giemsa |              | IHC |              | FISH | Note           |
|------------|--------|--------------|-----|--------------|------|----------------|
| 2896 cases | +      | 687 (23.7%)  | +   | 662 (22.8%)  | +    | 661            |
|            |        |              |     |              | -    | 1 1×1+         |
|            |        |              | -   | 25 (0.9%)    | +    | 4 4×1+         |
|            |        |              |     |              | -    | 21             |
|            | -      | 2209 (76.3%) | +   | 133 (4.6%)   | +    | 118            |
|            |        |              |     |              | -    | 15 14×1+; 1×2+ |
|            |        |              | -   | 2076 (71.7%) | +    | 5 5×1+         |
|            |        |              |     |              | -    | 2071           |

**TABLE 1** Summarized results of the studied stainings. Number and bacterial density of IHC-FISH discrepant cases are indicated in the "Note" column (eg, 4×1+ means four cases with 1+ grade of bacterial density)

specificity was 99.2%. Positive and negative *H. pylori* predictive values were 98.0% and 99.6%, respectively.

Regarding the equally high diagnostic accuracy of both IHC and FISH, a case was considered as *H. pylori* positive if *H. pylori* was detected by either IHC or FISH. Thus, 804 cases revealed *H. pylori* positivity (27.8%).

### 3.2 | Concordance and discrepancies between Giemsa, IHC, and FISH results

Analyzing the concordance and discrepancies between Giemsa, IHC, and FISH, 687 cases proved to be Giemsa positive. In the Giemsa-positive cohort, 661 of 662 cases (99.9%) were IHC and FISH positive. In the one remaining case, small foci of *H. pylori* colonization (1+) detected by Giemsa and IHC were carved out during serial sectionings, and hence, the FISH was negative in this specimen. Examining the 25 Giemsa-positive and IHC-negative cases, four proved to be FISH positive (1+) and 21 were found to be FISH negative. False positivity of Giemsa was typically caused by coccobacilli arranged in a shape resembling a coil (Figure 2E).

Considering the Giemsa-negative cases, 118 (5.3% of the 2209) were positive by both IHC and FISH. In two of these cases, coccoid form of *H. pylori* was shown by IHC and FISH (Figure 2F). Furthermore,

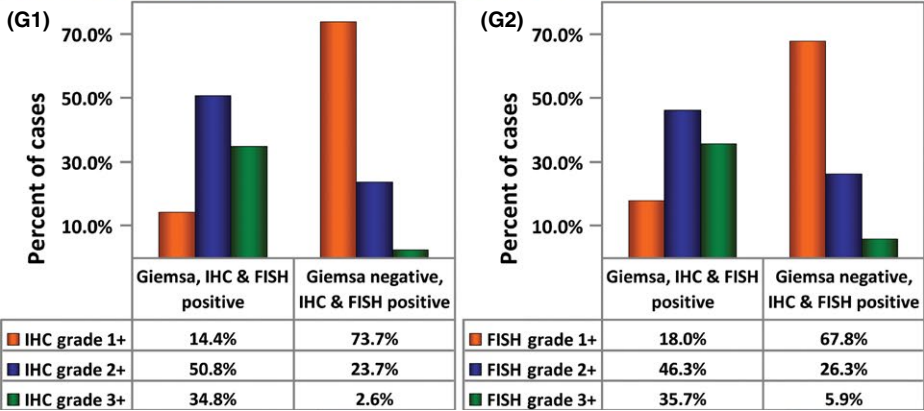
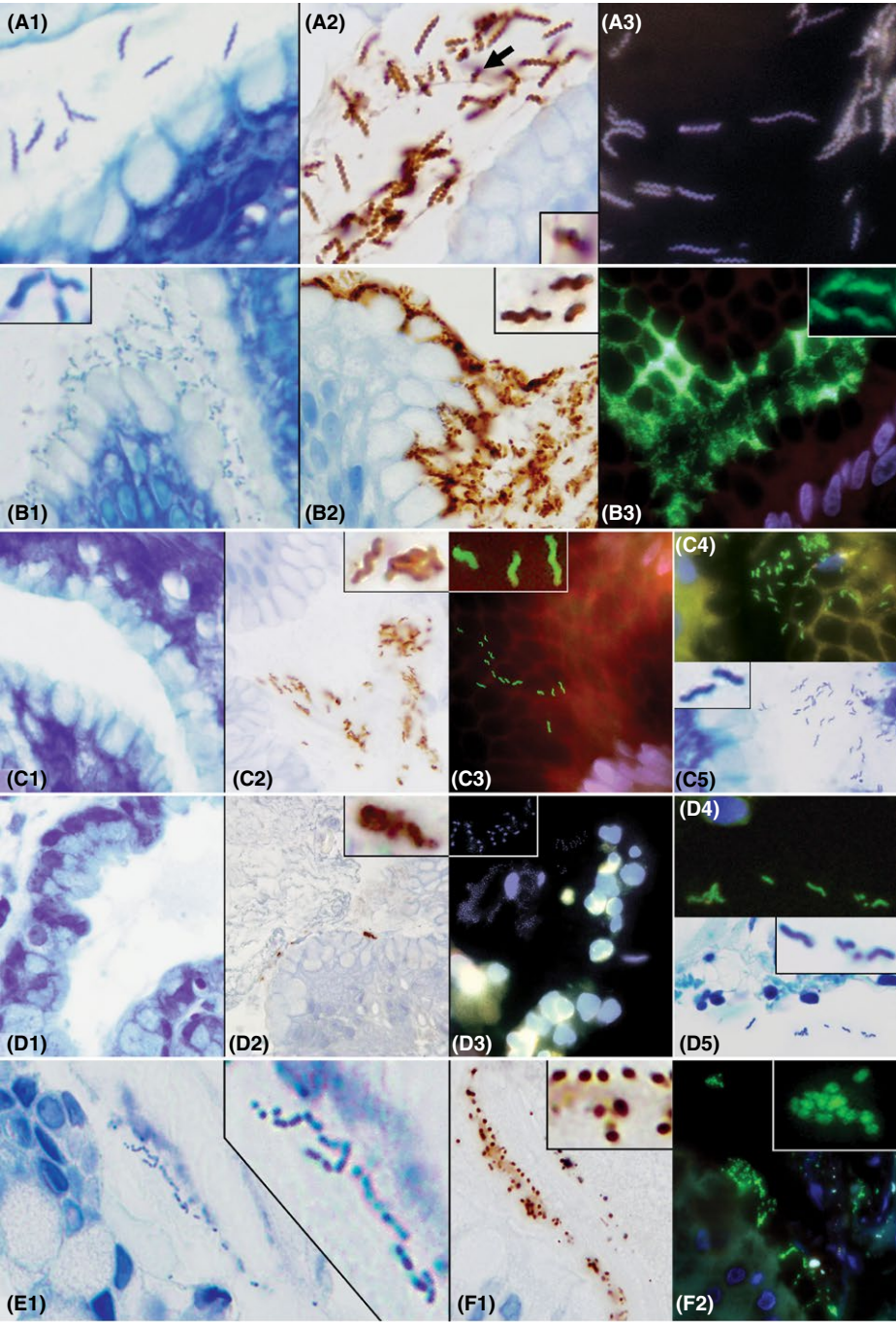
15 only IHC-positive and five only FISH-positive cases were detected. It is worthy of note that 2071 Giemsa-, IHC- and FISH-negative cases were found. More details are given in Table 1, Figure 2C, D.

Discrepancies between Giemsa, IHC, and FISH were examined by FISH-based re-staining of the Giemsa slides in 56 cases. The number of FISH on Giemsa-positive cases was the following in the next five groups: Giemsa (-) IHC (-) FISH (+)  $n=0/2$ ; Giemsa (-) IHC (+) FISH (-)  $n=6/22$ ; Giemsa (-) IHC (+) FISH (+)  $n=2/4$ ; Giemsa (+) IHC (-) FISH (-)  $n=0/22$ ; Giemsa (+) IHC (+) FISH (-)  $n=3/6$  (Table S2).

### 3.3 | Role of activity, mucosal structural alterations, and bacterial density

Results of Giemsa, IHC, and FISH stainings in the different statistical classes of *H. pylori*-positive cases are detailed in Tables 2 and 3. The positivity rate of Giemsa showed a considerable decrease to 23-44% in classes 0, 1, and 3 (classes without activity) (Table 2). In accordance with this, a highly significant difference of Giemsa sensitivity was found between the active (classes 2 and 4) and nonactive (classes 1 and 3) chronic gastritis cases (92.6% vs. 33.6%,  $P<.0001$ , Table 3). Although sensitivity of both IHC and FISH also showed a slight but statistically significant decrease in nonactive chronic gastritis, it remained high (over 90%) in terms of diagnostic requirements (classes

**FIGURE 2** Results of the Giemsa, IHC, and FISH staining. (A) *Helicobacter heilmannii*-infected case by different stainings: A1) Giemsa, A2) IHC, A3) FISH. *H. heilmannii* was identified by its characteristic morphology (longer, more tightly coiled spiral bacterium than *Helicobacter pylori*) as well as by bacteria showing dark brown positive staining by anti-*Helicobacter* IHC and showing negativity by *H. pylori* FISH (detectable only by autofluorescence, *H. pylori* specific green fluorescence is absent). Arrow and inset in A2: Partially visible *H. heilmannii* with shape resembling *H. pylori*. Original magnification: 1000×. (B) *H. pylori*-infected case with high bacterial density (3+). Positive staining by both Giemsa (B1; dark blue-stained bacteria), IHC (B2; dark brown positivity), and FISH (B3; green fluorescence). Original magnification: 1000×. (C) *H. pylori*-infected case with discrepant staining results: Giemsa negative (C1), IHC 2+ positive (C2; dark brown bacteria), FISH 1+ positive (C3; green fluorescent bacteria). Note the lower number of *H. pylori* bacteria in the section plane of FISH due to the multiple sectionings and the consequently decreased mucosal surface. FISH examination of the negatively diagnosed original Giemsa-stained slide revealed *H. pylori* positivity (C4; green fluorescent bacteria). Then, the slide was re-stained by Giemsa and re-evaluated, and thus, foci of *H. pylori* bacteria were also found by Giemsa (C5; dark blue-stained bacteria) in the localization indicated by FISH on Giemsa. Original magnification: 1000×. (D) *H. pylori*-infected case with discrepant staining results: Giemsa negative (D1), IHC 1+ positive (D2; dark brown bacteria), none of the few *H. pylori* bacteria were present in the section plane of FISH (D3) but other, coccoid bacteria (inset of D3, specific green fluorescence is absent). FISH examination of the negatively diagnosed original Giemsa-stained slide revealed *H. pylori* positivity (D4; green fluorescent bacteria). Then, the slide was re-stained by Giemsa and re-evaluated, and thus, few *H. pylori* bacteria were also found by Giemsa (D5; dark blue-stained bacteria) in the localization indicated by FISH on Giemsa. Original magnification: 1000×. (E) False *H. pylori* positivity of Giemsa was typically caused by coccobacilli (see in higher magnification in the inset) arranged in a shape resembling a coil. Original magnification: 630×. (F) Coccoid form of *H. pylori* by IHC (F1) and by FISH (F2). Original magnification: 1000×. (G) Distribution of *H. pylori* density results by IHC (G1) and by FISH (G2): Low-density (1+) infections were present in remarkably significantly higher proportion among the Giemsa negative but IHC- and FISH-positive cases, unlike those which were positive by all three stainings. (see the detailed numerical results in Table 4)



**TABLE 2** Results of the studied stainings by statistical classes in the *Helicobacter pylori*-positive cases. Greatly decreased proportions of positive cases are outlined in bold

| <i>H. pylori</i> -positive cases (n=804)                  |   | Giemsa   |           | IHC      |             | FISH     |             |
|-----------------------------------------------------------|---|----------|-----------|----------|-------------|----------|-------------|
| Statistical class                                         |   | -        | +         | -        | +           | -        | +           |
| No chronic gastritis                                      | 0 | 6 (67%)  | 3 (33%)   | 1 (12%)  | 8 (88%)     | 1 (12%)  | 8 (88%)     |
| Chronic nonactive gastritis without structural alteration | 1 | 36 (56%) | 28 (44%)  | 1 (1.5%) | 63 (98.5%)  | 4 (6%)   | 60 (94%)    |
| Chronic active gastritis without structural alteration    | 2 | 32 (7%)  | 433 (93%) | 3 (0.6%) | 462 (99.4%) | 0 (0%)   | 465 (100%)  |
| Chronic nonactive gastritis with structural alteration    | 3 | 47 (77%) | 14 (23%)  | 3 (5%)   | 58 (95%)    | 8 (13%)  | 53 (87%)    |
| Chronic active gastritis with structural alteration       | 4 | 17 (8%)  | 188 (92%) | 1 (0.5%) | 204 (99.5%) | 3 (1.5%) | 202 (98.5%) |

**TABLE 3** Comparison of sensitivity of the studied staining techniques in the different statistical classes of *Helicobacter pylori*-positive cases and their combinations (two-tailed McNemar's test; CI of OR: 95% confidence interval of odds ratio; NaN: not a number). Greatly decreased sensitivities appear in bold cell borders. In case of nonsignificant difference, *P* values are indicated in italics and CI of OR is absent

| <i>H. pylori</i> -positive cases (n=804)                  |   |     | Sensitivity (%) |       |        | Comparison of sensitivity ( <i>P</i> value/<br>CI of OR) |                           |          |
|-----------------------------------------------------------|---|-----|-----------------|-------|--------|----------------------------------------------------------|---------------------------|----------|
| Statistical class                                         | n |     | Giemsa          | IHC   | FISH   | Giemsa-IHC                                               | Giemsa-FISH               | IHC-FISH |
| Chronic nonactive gastritis without structural alteration | 1 | 64  | 43.8%           | 98.4% | 93.8%  | <0.000001<br>3.81-205.8                                  | <0.000001<br>2.83-22.6    | 0.375000 |
| Chronic active gastritis without structural alteration    | 2 | 465 | 93.1%           | 99.4% | 100.0% | <0.000001<br>3.71-64.8                                   | <0.000001<br>NaN-Infinity | 0.250000 |
| Chronic nonactive gastritis with structural alteration    | 3 | 61  | 23.0%           | 95.1% | 86.9%  | <0.000001<br>NaN-Infinity                                | <0.000001<br>NaN-Infinity | 0.226563 |
| Chronic active gastritis with structural alteration       | 4 | 205 | 91.7%           | 99.5% | 98.5%  | 0.000145<br>2.26-127.8                                   | 0.000519<br>1.98-113.6    | 0.625000 |
| Combination of 1,3                                        |   | 125 | 33.6%           | 96.8% | 90.4%  | <0.000001<br>NaN-Infinity                                | <0.000001<br>NaN-Infinity | 0.076813 |
| Combination of 2,4                                        |   | 670 | 92.6%           | 99.4% | 99.6%  | <0.000001<br>4.98-51.4                                   | <0.000001<br>6.48-340.7   | 1.000000 |
| Combination of 1,2                                        |   | 529 | 87.1%           | 99.2% | 99.2%  | <0.000001<br>8.08-134.7                                  | <0.000001<br>NaN-Infinity | 1.000000 |
| Combination of 3,4                                        |   | 266 | 75.9%           | 98.5% | 95.9%  | <0.000001<br>8.46-440                                    | <0.000001<br>7.47-390.3   | 0.118469 |
| Combination of 1,2,3,4                                    |   | 795 | 83.4%           | 99.0% | 98.1%  | <0.000001<br>13.5-133                                    | <0.000001<br>16.5-844.7   | 0.210040 |
| Combination of 0,1,2,3,4                                  |   | 804 | 83.3%           | 98.9% | 98.0%  | <0.000001<br>12.3-89.9                                   | <0.000001<br>17.2-880.2   | 0.229523 |

1&3 vs 2&4: 96.8% vs 99.4%;  $p=0.0245$  for IHC and 90.4% vs. 99.6%;  $p=4.26 \times 10^{-8}$  for FISH, Table 3).

McNemar's test revealed highly significant ( $P<.001$ ) differences in the sensitivity values comparing Giemsa to both IHC and FISH in all four classes of chronic gastritis, while no significant differences were found between the performances of IHC and FISH (Table 3). *H. pylori* detection ability of the three staining techniques was evaluated separately by statistical classes via Holm-Bonferroni method. No specific pattern of statistically similar and different classes was found for IHC and FISH.

However, Giemsa-based *H. pylori* detection showed an activity-specific pattern, as no statistically significant differences were observed between classes 1 and 3 (nonactive classes) as well as between classes 2 and 4 (classes with activity), whereas sensitivity values differed significantly from each other in all other class combinations. Therefore, interestingly, inflammatory activity turned out to be the primary factor influencing Giemsa sensitivity (more data and *P* values shown in Table 3).

Impaired sensitivity of Giemsa correlated with the low density of *H. pylori* as the majority of Giemsa false-negative cases showed

**TABLE 4** Distribution of infection densities of 1+ (+), 2+ (++), and 3+ (+++) in different subgroups of the *Helicobacter pylori*-positive cases

|                                      | n       | Grade of the <i>H. pylori</i> infection (semiquantitatively graded) |               |             | IHC/FISH |
|--------------------------------------|---------|---------------------------------------------------------------------|---------------|-------------|----------|
|                                      |         | +                                                                   | ++            | +++         |          |
| Total IHC positive                   | 795     | 24.8% (197)                                                         | 45.9% (365)   | 29.3% (233) | IHC      |
| Total FISH positive                  | 785     | 26.1% (208)                                                         | 42.9% (337)   | 31% (243)   | FISH     |
| Giemsa, FISH & IHC positive          | 661     | 14.4% (95)                                                          | 50.8% (336)   | 34.8% (230) | IHC      |
|                                      |         | 18% (119)                                                           | 46.3% ((306)) | 35.7% (236) | FISH     |
| Giemsa negative, FISH & IHC positive | 118     | 73.7% (87)                                                          | 23.7% (28)    | 2.6% (3)    | IHC      |
|                                      |         | 67.8% (80)                                                          | 26.3% (31)    | 5.9% (7)    | FISH     |
| Statistical class 1                  | 63/64   | 43.8% (28)                                                          | 48.4% (31)    | 6.3% (4)    | IHC      |
|                                      | 60/64   | 51.6% (33)                                                          | 35.9% (23)    | 6.3% (4)    | FISH     |
| Statistical class 2                  | 462/465 | 17.2% (80)                                                          | 49.2% (229)   | 32.9% (153) | IHC      |
|                                      | 465/465 | 19.8% (92)                                                          | 44.3% (206)   | 35.9% (167) | FISH     |
| Statistical class 3                  | 58/61   | 65.6% (40)                                                          | 26.2%(16)     | 3.3% (2)    | IHC      |
|                                      | 53/61   | 55.7% (34)                                                          | 21.3% (13)    | 9.8% (6)    | FISH     |
| Statistical class 4                  | 204/205 | 20.5% (42)                                                          | 42.9% (88)    | 36.1% (74)  | IHC      |
|                                      | 202/205 | 21.5% (44)                                                          | 45.4% (93)    | 31.7% (65)  | FISH     |

1+ bacterial density infection by both IHC (1+: 73.7%) and FISH (1+: 67.8%), while antagonistic tendency was observed among the cases positive by Giemsa too, where a significantly lower number of 1+ grade infections was detected by both IHC (14.4%) and FISH (18%) ( $P < .0001$ , Table 4, Figure 2G).

Although activity had only a slight effect on the performance of IHC and FISH, the distribution of 1+, 2+, 3+ bacterial density infections (Table 4) strongly correlated with the presence or absence of activity. Namely, grade distribution pattern showed no significant differences between classes 1 and 3 as well as between classes 2 and 4, but comparisons of classes in all the other possible combinations showed highly significant differences ( $P < .0001$  by both IHC and FISH;  $P < .01$  with Holm-Bonferroni method for multiple comparison).

Accordingly, density grade 1+ was observed significantly more frequently in patients showing no activity (classes 1 and 3) by both IHC and FISH ( $P < .0001$ , respectively) and an activity-specific pattern was observable upon multiple comparison using the Holm-Bonferroni method (no significant differences in class 1 to 3 and 2 to 4 comparisons, but  $P \leq .0002$  by IHC and  $P < .0001$  by FISH in all other combinations of classes) (Table 4).

The presence of structural alterations of the gastric mucosa had only a limited effect on the diagnostic performance of the three stainings. Namely, comparison of cases with and without structural alterations (classes 1&2 vs 3&4, Table 3) did not show significant differences regarding sensitivity of IHC (99.2% vs 98.5%,  $P = .4520$ ). Furthermore, a slight but statistically significant decrease in FISH sensitivity (98.0% vs 95.9%,  $P = .0017$ ) and a slightly larger impairment of Giemsa sensitivity (87.1% vs 75.9%,  $P < .0001$ ) were observed due to the mucosal structural alterations. Each of these alterations (including intestinal metaplasia) had a statistically nonsignificant or only slightly negative effect on the sensitivity of the different stainings (data not shown).

CART analysis identified Giemsa as the best discrimination factor (except for IHC and FISH) of *H. pylori*-positive cases and demonstrated

that none of the other variables (histologic features) could further improve the diagnostic efficiency. Therefore, false prediction rates of Giemsa in the different statistical classes may indicate the necessity of an additional *H. pylori* staining, such as IHC or FISH. Accordingly, false prediction rate (combined proportion of false-negative and false-positive cases) of Giemsa showed significant differences between the different statistical classes of the total included population ( $n = 2896$ ), (Class 0: 6/744 (0.8%), Class 1: 40/853 (4.7%), Class 2: 38/575 (6.6%), Class 3: 50/422 (11.8%), Class 4: 25/302 (8.3%);  $P < .0001$ ; more details are given in Table S3 in the Supplementary Appendix).

## 4 | DISCUSSION

Special stains (including Giemsa) are known to be the most widely applied techniques for histopathologic detection of *H. pylori*. Correspondingly, 29 of 60 Hungarian pathology departments (48%) use Giemsa as routine *H. pylori* stain, other special stains are used by further 15 departments (25%), while the share of the more advanced IHC is only 27% (Kocsmár et al., unpublished data).

In the recent years, utility of different stains for detection of *H. pylori* has been intensively studied and discussed.<sup>14-17</sup> However, the relatively small number of cohorts (varying between 56 and 356 patients) is a possible major limitation of these studies. With 2896 patients included, ours is one of the largest single center studies comparing the performance of *H. pylori* immunohistochemistry and a conventional staining, as well as the largest one investigating the *H. pylori* FISH technique.

Considering the results of the above-mentioned reports, special stains do not necessarily perform better than H&E staining, while immunohistochemistry is suggested to be the best staining method. Still, it was concluded that H&E-stained sections are usually sufficient to detect *H. pylori*.<sup>14-17</sup> On the contrary, our large-scale

cohort clearly proved that the conventional staining techniques have remarkable disadvantages. Giemsa showed a significantly lower overall sensitivity, and its performance was strongly influenced by inflammatory activity resulting a dramatic impairment in the non-active cases (Table 3). Interestingly, mucosal structural alterations including intestinal metaplasia had only a minor sensitivity-reducing effect. On the other hand, sensitivity of both IHC and FISH remained high even in the nonactive cases (Table 3). In the background of the above, we found a strong positive correlation between *H. pylori* density and inflammatory activity strongly suggesting that low sensitivity of Giemsa staining observed in absence of activity is related to low bacterial density. However, this factor much less influenced overall sensitivity of either IHC or FISH.

Insufficient diagnostic performance of Giemsa was also revealed through re-staining the original Giemsa slides by FISH. This identified false *H. pylori*-positive result of Giemsa (usually caused by coccobacilli; Figure 2E) in each of the 22 IHC-FISH double-negative and in half of the six Giemsa and IHC positive but FISH-negative cases. On the other hand, FISH on Giemsa detected *H. pylori* in the Giemsa-negative slide in eight of 28 IHC- and/or FISH-positive cases, with 1+ *H. pylori* density in seven of these. This finding further supports that Giemsa is not sensitive enough, especially in cases with low *H. pylori* density. Moreover, Giemsa failed to differentiate between coccoid *H. pylori* bacteria and other cocci, leading to false-negative results in certain cases (Figure 2F).

Our results confirm that IHC and FISH have equally high sensitivity both in the total studied population and in any of the histopathologic subgroups (Table 3). However, an important limiting factor of the *H. pylori* stainings should be discussed here. Namely, *H. pylori* shows an uneven and patchy distribution on the gastric mucosa, especially in case of low bacterial density.<sup>18,19</sup> The consequences of this can be highlighted by analyzing the IHC-FISH discrepant cases of our study. In 24 of the 25 IHC-FISH discrepant cases, only mild (1+) *H. pylori* density was identified, frequently only with scattered foci of few bacteria (Figure 2D). For example, in one of these cases, just three(!) single *H. pylori* bacteria were detected in only one of the three tissue sections mounted on the IHC slide. In these cases, sparse smaller groups of *H. pylori* or individual bacteria were probably carved either out or in during the repeated sectionings.

This was confirmed by FISH on Giemsa results of the Giemsa-negative IHC-FISH discrepant cases. FISH verified the *H. pylori* negativity of 20/28 Giemsa-stained slides, surprisingly including four cases where the original FISH slide was *H. pylori* positive. This further supports that scattered distribution of the bacteria can cause false negativity of any of the *H. pylori* stainings, but it can also lead to the appearance of new *H. pylori* containing foci at deeper cutting levels. The occurrence of such cases could be reduced by multiple biopsy sampling and by investigating multiple sections from each sample.

Another important issue is that the less the surface epithelium in a gastric tissue sample, the lesser the chance of finding *H. pylori* bacteria. As, contrary to IHC, only a single section from the last (third) sectioning course was used for FISH, reduction of the surface epithelium due to repeated sectionings led to significantly decreased or even totally

lost *H. pylori* content in certain FISH slides. Hence, in our study, the slightly (statistically not significant) lower sensitivity of FISH (Table 3) in comparison with IHC is rather an issue of the somewhat higher level of this special kind of sampling error, and not the consequence of a lower analytical sensitivity.

As molecular techniques are becoming widely available, the advantages and need for these novel methods should be determined for novel diagnostic algorithms. *H. pylori* FISH is also available in commercial kits (BACTFish, Izinta Kft., Budapest, Hungary; Probe4Pylori, Biomode SA, Caldas das Taipas, Portugal), and as we have shown, its sensitivity does not differ significantly from the known high sensitivity of IHC. However, in contrary to FISH, IHC gives cross-reaction with other *Helicobacters*, such as *Helicobacter heilmannii*.<sup>20,21</sup> This can lead to misinterpretation of IHC results (Figure 2A) establishing a false diagnosis of mixed *H. heilmannii* and *H. pylori* infection that can be excluded by FISH.

*H. pylori* FISH technique has a further advantage along with the *H. pylori* detection, as it can determine the clarithromycin susceptibility-resistance status of the bacteria as well.<sup>10,22</sup> This is of high therapeutic importance as clarithromycin is the basic and most effective drug of first-line *H. pylori* treatment. If the incidence of primary resistance to clarithromycin reaches 15% in a population, other first-line eradication protocols or testing for the clarithromycin susceptibility of *H. pylori* infections are recommended.<sup>4</sup> Hence, it is important to examine the susceptibility/resistance status at population level and, if required, at personal level as well.

Routine diagnostics of *H. pylori* seems to be of lesser importance for certain pathologists probably because of its simplicity. However, primary prevention of peptic ulcer, MALT lymphoma, or gastric cancer depends on the correct diagnosis. Accordingly, high sensitivity of the chosen *H. pylori* detection method is of great importance because the bacterial load and inflammatory activity may decrease in certain conditions, for example, either following antibiotic treatment or proton-pump inhibitors, or in antral atrophy. Glickman et al. also found that nonimmunohistochemical stains are unreliable for the detection of rare *H. pylori* bacteria in these cases, where organisms are commonly located in deep oxyntic glands or within the canaliculi of parietal cells.<sup>23</sup> However, long-time complications of *H. pylori* infection, such as chronic ulceration, atrophy, intestinal metaplasia, or gastric cancer may develop in the presence of reduced bacterial number, too.<sup>4,14</sup> In order to avoid medical liability issues due to false-negative diagnosis of *H. pylori*, it is important to detect *H. pylori* irrespective of low bacterial density or other aggravating conditions.

In their editorial article, Yantiss and Lamps conceived the need for further studies investigating the value of conventional stains in unselected populations including patients with minimal inflammatory disease.<sup>14</sup> In response to this, our study showed that the sensitivity of Giemsa staining depends strongly on the *H. pylori* density and hence on the presence of activity. Contrarily, sensitivity of both IHC and FISH is minimally influenced by bacterial density and mucosal changes. Moreover, both are capable of detecting uncommon, rare forms (eg, coccoid form) of *H. pylori*, which is hardly possible by means of the conventional stainings.

Based on our presented results, we highly recommend immunohistochemistry for *H. pylori* detection basically in clinically susceptible, histologically nonactive chronic gastritis cases, if the H&E- and/or ancillary stain-based *H. pylori* detection is negative. On the other hand, considering not only the false-negative but also the *H. pylori* false-positive cases, the highest false prediction rates of Giemsa occurred if mucosal structural alterations were present. Therefore, the use of the more accurate IHC can be beneficial in these cases, independently of the presence or absence of inflammatory activity.

However, it is worth considering that the conventional stainings are answers of the 1980s to the challenges of *H. pylori* detection. The state-of-the-art solution to this problem is immunohistochemistry. This was clearly demonstrated by Lash and Genta in their recent large-scale study reporting that the use of upfront immunohistochemical stains resulted in a dramatic improvement in detection of *H. pylori* infection.<sup>24</sup> It would therefore be desirable to use the IHC technique more widely as a basic *H. pylori* stain. Moreover, in the era of personalized medicine, *H. pylori* FISH should be used in verified *H. pylori*-positive cases to determine the clarithromycin susceptibility of *H. pylori* before administering first-line eradication treatment in populations with high clarithromycin resistance rates.

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## DISCLOSURES

The authors declare no conflict of interest.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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