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Fluorescence diagnostics of Helicobacter pylori-infected human gastric mucosa: Establishing technique and validity

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Abstract

Objective. Bacterial factors, including strain type, anatomic distribution and density, and host responses are important determinants in the pathogenesis of erosive and neoplastic changes linked to gastric Helicobacter pylori (H. pylori) infection. The purpose of this study was to investigate the potential use of photodiagnostics in mapping H. pylori infection. The relationship between fluorescence in individual gastric pits of H. pylori (+) and H. pylori (−) subjects versus that in larger field views of the gastric mucosa and the use of fluorescence to determine H. pylori status in different gastric areas were studied. Material and methods. Antrum, corpus and fundus biopsies from 8 H. pylori (+) and 4 H. pylori (−) subjects taken during two gastroscopies were used for autofluorescence (535 nm excitation) and aminolevulinic acid (ALA)-induced protoporphyrin IX fluorescence (405 nm excitation) determinations. Results. In the antrum, corpus and fundus a close correlation between individual pit and full-field image (FFI) fluorescence was demonstrated for H. pylori status (R > 0.85; R > 0.75; R > 0.80) and both excitation wavelengths (R > 0.89; R > 0.85; R > 0.95), respectively. In the antrum, FFI in H. pylori (+) subjects exceeded that in H. pylori (−) subjects using 405 nm but not 535 nm excitation regardless of ALA treatment (R ≤ 0.026). In the corpus and fundus, fluorescence using 405 nm excitation was greater in H. pylori (+) than in H. pylori (−) subjects only after ALA treatment (p < 0.00005, p = 0.03). The ratio of 535 nm:405 nm-excited fluorescence decreased from the fundus > corpus > antrum for both H. pylori (+) and H. pylori (−) subjects regardless of ALA treatment (p = 0.03). Conclusions. Fluorescence-based identification of areas of H. pylori-infected gastric mucosa using 405 nm excitation combined with ALA treatment is feasible and, using a ratio of 535 nm:405 nm-excited fluorescence, it is possible to distinguish H. pylori status and the different areas of the stomach even without ALA.

Key Words: Aminolevulinic acid, autofluorescence, gastritis, Helicobacter pylori, human, laser, photodynamic diagnosis, photosensitizer, stomach

Introduction

Helicobacter pylori (H. pylori) is a common Gram-negative, flagellated bacterium with strict tropism for gastric mucosa that is found in over 50% of the world's population. It is predominantly localized in the mucous layer in the crypts of the gastric glands in microaerobic conditions. H. pylori infection causes chronic gastritis, which can lead to peptic ulceration or in approximately 50% to gastric atrophy, and further to metaplasia, dysplasia and, in a small percentage, it is implicated in gastric neoplasia [1]. Density and localization of H. pylori colonization, type of H. pylori strains and host response, as well as other non-H. pylori-related conditions, are known to influence the course of a patient's gastric disease development [2–7]. Although eradication using a combination of antibiotics and acid inhibitors is generally effective, antibiotic resistance and recurrence of infection are not uncommon.

Currently, clinicians lack the ability to map the location, severity and type of H. pylori infection accurately and non-invasively. Owing to its invasiveness and cost, biopsy/histopathological evaluation can only be performed in a limited number of sites in any patient and the results provide information only
on the specific sites sampled, which may not be representative [8]. Although non-invasive methods for the diagnosis of the general presence of *H. pylori* such as the $^{13}$C-urea breath test, serological or stool tests are available [9], localization, density mapping and a dynamic visualization of the area of infection are not possible and would be useful tools to increase our understanding of the pathogenesis and ecology of *H. pylori*-related disease.

Photodynamic diagnosis (PDD) is a technique based upon the specific and selective fluorescence of accumulated photosensitizers in areas of dysplasia or malignancy. PDD using 5-aminolevulinic acid (5-ALA), a commonly used precursor of the photosensitizer protoporphyrin IX (PPIX), is an effective method for identifying dysplasias and malignant lesions in the gastrointestinal tract [10]. 5-ALA has been shown to induce significant photodynamic effects in both Gram-positive and Gram-negative bacteria, including *H. pylori* [11,12]. Past in vitro studies with 5-ALA have demonstrated potent photosensitization of *H. pylori*, with kill rates of >95% indicating that the organism accumulates significant amounts of PPIX [13]. Our previous investigation using fluorescence-based diagnostics of biopsy material has shown that it is possible to identify individual gastric pits containing *H. pylori* after ingestion of ALA [14]. It would, therefore, seem possible to detect the presence of *H. pylori* using PDD during gastroscopy after the ingestion of ALA by infected individuals.

The goals of this study were to investigate the potential use of photodiagnostics in mapping *H. pylori* infection. For this purpose 1) the relationship between fluorescence in individual gastric pits of *H. pylori* (+) and *H. pylori* (−) subjects versus that in larger field views of the gastric mucosa and 2) the use of fluorescence to determine *H. pylori* status in different gastric areas were studied. For the sake of convenience, we have referred to fluorescence emissions at 535 nm excitation as “autofluorescence” and to those after excitation at 405 nm as “ALA-induced PPIX fluorescence.” PPIX has a strong absorption peak at 408 nm [15]. In ALA-treated tissues, although the majority of the fluorescence emissions above 610 nm will be due to ALA-induced PPIX fluorescence, some additional endogenous emissions will also contribute to the total light emitted [16,17]. Similarly, “autofluorescence” in ALA-treated tissues will include a small amount of emissions from PPIX, which has weak absorption near the 535 nm excitation [15].

### Material and methods

#### Subjects

Eight asymptomatic *H. pylori*-positive volunteers (5 M, 3 F, mean age 35 years, range 21–48 years) and four *H. pylori*-negative volunteers (1 M, 3 F, mean age 31 years, range 22–35 years) were recruited. Exclusion criteria were a history of gastrointestinal, hepatic or dermatological disease, coagulation disorders, any medication in the past 14 days, pregnancy, lactation and porphyria. Before the start of the study *H. pylori* infection was confirmed or excluded in antrum, corpus and fundus biopsies by histology using Giemsa and immunohistochemical staining (Novocastra Laboratories Ltd., Newcastle, England), the rapid urease biopsy test (HUT; AstraZeneca, Zug, Switzerland), *H. pylori* culture, and by the $^{13}$C urea breath test [9]. Biopsies for *H. pylori* culture, histology and urease testing were obtained using a different biopsy forceps from the antrum, corpus and fundus. The study was approved by the institutional ethics committee and all subjects gave written informed consent before the start of the study.

Gastric antrum, corpus and fundus biopsies were obtained during each of two separate gastroscopies, performed within four to nine days of each other. Biopsies from the first gastroscopy without 5-ALA ingestion were used for autofluorescence mapping. The biopsies from the second gastroscopy, taken 30 min after ingestion of 20 mg/kg ALA (ALAT AG, Zug, Switzerland) in 200 ml of orange juice, were used for ALA-induced PPIX fluorescence mapping. After ALA ingestion, subjects rotated onto their left and right sides for 20 min to ensure an even distribution of the sensitizer. Lansoprazole 30 mg (Takeda Pharma, Lachen, Switzerland) was taken per os on the evening and morning before each gastroscopy to stabilize pH and prevent ALA degradation. Subjects received midazolam (2–5 mg) and scopolamine butylbromide (20 mg) i.v. before the gastroscopy. Subjects were observed for 30 min after the end of each study procedure and were required to shield themselves from direct sunlight for 24 h post-ALA treatment.

#### Fluorescence measurements

Two 2-mm pinch biopsies were taken from each area for histology. The specimens were immediately placed in Tissue-Tek OCT compound (Sakura Finetek, Europe B.V., NL) and flash-frozen in liquid nitrogen. All biopsies were shielded from light and stored at −70°C until histological processing. Twelve micron-thick cryosections were cut under
subdued lighting, mounted on cover glasses and stored at −70°C until imaged. The fluorescence images were captured within 48 h of sectioning using a 100 × oil objective on a Zeiss Axiomat microscope interfaced with a Princeton Instruments CCD camera and a Macintosh computer running IPLab (Scanalytics Inc., Fairfax, Va., USA) software. Excitation occurred at 405 nm, an absorption maximum for both PPIX [15] and *H. pylori* [18], and at 535 nm, another absorption peak for *H. pylori* [18] but not most endogenous tissue components [19]. A 100 W mercury lamp source combined with narrow-band pass filters (Chroma Technology Corp., Brattleboro, Vt., USA) produced the excitation light. Only the fluorescence emissions above 610 nm were recorded. After imaging, the sections were heat fixed and stained using the modified Giemsa method [9] for *H. pylori* confirmation. Glands previously imaged by fluorescence microscopy were relocated and photographed using a 100 × oil objective on an Olympus BHTU microscope equipped with an Olympus Microfire digital camera and software.

**Data analysis**

A minimum of four separate areas from at least three different sections of each biopsy specimen were examined. Separate fluorescence images were obtained for each excitation wavelength. All images were analyzed using IPLab software. Calibration images were taken on each capture date and all fluorescence data normalized to those of a single reference date.

In a subset of samples from all the gastric areas, both individually traced pit areas and the full-field image (FFI) containing the individual pits were analyzed. The mean fluorescence intensities were determined in arbitrary units, A.U./pixel ± SD. The pit and full-field values were compared to determine whether the fluorescence intensity measurements were the same, different or correlated. Correlation coefficients and their probabilities were calculated for each data set. Scatter plots for each data set were examined to determine data spread around their respective regression lines.

The mean FFI fluorescence for each of the images captured from all the gastric samples was determined. This was expressed as mean A.U./mm² of area visualized to reflect the larger area examined. Data were pooled according to the combined factors of *H. pylori* status, ALA treatment, gastric location and excitation wavelength. A mean A.U./mm² ± SD was determined for each of the 24 resulting data sets. For both excitation wavelengths at each gastric location, t-tests comparing *H. pylori* (+) versus *H. pylori* (−) subjects both with and without ALA treatment were performed. Two-way ANOVA tests for interaction of *H. pylori* status and ALA treatment were also performed for each gastric location and both excitation wavelengths. A level of significance of *p* < 0.05 was used.

The mean ratio of FFI autofluorescence to FFI ALA-induced fluorescence ± SD was calculated at each gastric location for both *H. pylori* conditions, with and without ALA treatment. Two-way ANOVA and least squares analysis of the means were performed for each data set to test for interaction of *H. pylori* status, ALA treatment and gastric location. A *p*-value of less than 0.05 was considered as significant.

**Results**

Figure 1 shows a typical gastric gland from the corpus of a *H. pylori*-positive subject. The field was phase imaged (A) prior to fluorescence excitation with 535 nm light (B) and 405 nm light (C). Tissue sections were Giemsa stained (D) following fluorescence imaging and pits were relocated to verify the presence or absence of *H. pylori* infection. All fields examined were similarly documented.

As shown in Table I, there is a strong correlation in all gastric areas between the individual pit values and the corresponding FFI fluorescence, whether comparing either infection status, *H. pylori* (+) versus *H. pylori* (−), or excitation wavelengths (all *p* < 0.0005). Figure 2 depicts the mean antral fluorescence intensities A.U./pixel ± SD of pits and corresponding FFI for *H. pylori* (+) and *H. pylori* (−) subjects with and without ALA treatment following excitation at 405 nm (A) and 535 nm (B). In all groups the FFI shows significantly higher fluorescence compared with individual pit values (all *p* < 0.0005). In pits treated with ALA and FFI, independently of ALA treatment the fluorescence was significantly higher (pit, *p* < 0.0005; FFI, *p* ≤ 0.03) at 405 nm excitation in *H. pylori* (+) than in *H. pylori* (−) samples. The pit and FFI values for the corpus and fundus are presented in Table II. Unlike the antrum, in the corpus and the fundus both pits and FFI from *H. pylori* (+) subjects had greater fluorescence than those from *H. pylori* (−) subjects using 405 nm excitation only after ALA treatment (pit, *p* ≤ 0.008; FFI, *p* ≤ 0.0009) Figure 3 is a representative scatter plot showing the close correlations between individual pit values and the corresponding FFI fluorescence seen for the *H. pylori* (+) (*r* = 0.84, *p* < 0.0005) and *H. pylori* (−) (*r* = 0.99, *p* < 0.0005) data samples with ALA treatment in the antrum. The scatter plots from all the correlation measurements comparing the fluorescence in the pits and the FFI containing them showed similarly tight clustering around their
Figure 1. Photomicrograph of a \(H.\) pylori-positive pit from the corpus. A. phase image before fluorescence excitation. B. Raw image during excitation with 535 nm light. C. Raw image during excitation with 405 nm light. B. & C. Long-pass filter of 610 nm used. D. Bright-field image of identical pit relocated after Giemsa staining to verify \(H.\) pylori status. The inset shows an area of \(H.\) pylori localization in the gastric pit.

respective regression lines whether grouped by \(H.\) pylori status, excitation wavelength or ALA treatment (data not shown).

Figure 4 displays FFI mean normalized fluorescence intensities (A.U./mm\(^2\)±SD) at 405 nm and 535 nm excitation of the antrum (A), corpus (B) and fundus (C) regions of \(H.\) pylori(+) and \(H.\) pylori(-) subjects before and after ALA administration. Data were compared for \(H.\) pylori(+) versus \(H.\) pylori(-) for both ALA and non-treated samples. With 405 nm excitation, fluorescence of \(H.\) pylori(+) samples was greater than that of \(H.\) pylori(-) samples for both ALA and non-ALA treated samples in the antrum (\(p \leq 0.026\)). In the corpus and the fundus the fluorescence at 405 nm of \(H.\) pylori(+) samples was significantly greater than that of \(H.\) pylori(-) samples only after ALA treatment (\(p < 0.00005\), \(p = 0.03\), respectively). At 535 nm excitation, \(H.\) pylori(-) fluorescence was greater than \(H.\) pylori(+) fluorescence in non-ALA treated samples for both the corpus and fundus (\(p = 0.005\), \(p = 0.049\), respectively). Fluorescence in the ALA-treated corpus and fundus samples at 535 nm excitation was similar for both \(H.\) pylori(+) and \(H.\) pylori(-) (\(p = 0.84\)). No significant differences in fluorescence emission intensities at 535 nm were identified between the ALA versus non-ALA treatment groups for either \(H.\) pylori status in the antrum (\(p \geq 0.11\)).

Figure 5 shows the mean ratio of the fluorescence for 535 nm:405 nm excitation for both \(H.\) pylori(+) and \(H.\) pylori(-) subjects with and without ALA treatment in the antrum, corpus and fundus. Significant differences were found between the ratio values from the different areas with values in the fundus > corpus > antrum for both \(H.\) pylori(+) and \(H.\) pylori(-) subjects (ANOVA: \(p = 0.03\)). For all areas, the ratios were highest for the \(H.\) pylori(-) subjects whether ALA treated or not. In the antrum and corpus, the ratio differences between \(H.\) pylori(-) and \(H.\) pylori(+) subjects were significant (\(p = 0.0001\), \(p < 0.00005\), respectively), whereas the fundus was borderline (\(p = 0.057\)) regardless of ALA treatment. In all three areas there was a decrease in the ratio for the \(H.\) pylori(-) subjects after ALA treatment, although it was only significant for the antrum (\(p = 0.04\)). Figure 6 shows the distribution of the ratios for each of the gastric areas by \(H.\) pylori status and ALA treatment. In the non-ALA-treated samples the range of values seen for the \(H.\) pylori(+) subjects generally extends below that of the \(H.\)

Table I. Correlation values for fluorescence in individual pits and the full frame images containing the pits.

<table>
<thead>
<tr>
<th>Combined excitation wavelengths</th>
<th>Combined (H.) pylori status</th>
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<tbody>
<tr>
<td></td>
<td>(H.) pylori(-)</td>
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<tr>
<td></td>
<td>ALA(-) ((n = 30))</td>
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<tr>
<td></td>
<td>ALA(+) ((n = 20))</td>
</tr>
<tr>
<td></td>
<td>ALA(-) ((n = 32))</td>
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<tr>
<td></td>
<td>ALA(+) ((n = 44))</td>
</tr>
<tr>
<td>Antrum</td>
<td>0.984 ((n = 41))</td>
</tr>
<tr>
<td>Corpus</td>
<td>0.992 ((n = 30))</td>
</tr>
<tr>
<td>Fundus</td>
<td>0.960 ((n = 60))</td>
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</tbody>
</table>

Abbreviation: ALA = aminolevulinic acid.

\(n\) = numbers of samples processed. All correlations are significant at \(p < 0.0005\).
pylori(−) subjects. After ALA treatment, there is a shift in the H. pylori(+) values such that they are contained within the range seen for the H. pylori(−) subjects. The sensitivity and specificity rates obtained when using different cut-off points to distinguish between H. pylori status with and without ALA treatment are reported in Table III.

None of the subjects reported significant side effects during the study. The procedures and the 5-ALA treatment were well tolerated.

Discussion

Autofluorescence and ALA-induced PPIX fluorescence were both well-visualized and readily quantified using validated techniques. A strong correlation between fluorescence in individual pit values and the corresponding FFI fluorescence was confirmed regardless of infection status or excitation wavelengths. The substantially lower mean fluorescence

Table II. Fluorescence values for individual pits and the full-frame images containing the pits from the corpus and fundus.

<table>
<thead>
<tr>
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<th>405 nm Excitation</th>
<th>535 nm Excitation</th>
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<td></td>
<td>ALA(−)</td>
<td>ALA(+)</td>
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<tr>
<td>H. pylori(+)</td>
<td>4.0±2.5</td>
<td>3.1±2.5</td>
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<td>H. pylori(−)</td>
<td>14.6±2.5</td>
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Corpus

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<th>Pit</th>
<th>Full frame</th>
<th>Pit</th>
<th>Full frame</th>
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<td>(n=28)</td>
<td>(n=10)</td>
<td>(n=27)</td>
<td>(n=15)</td>
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Fundus

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<thead>
<tr>
<th></th>
<th>Pit</th>
<th>Full frame</th>
<th>Pit</th>
<th>Full frame</th>
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<tr>
<td>(n=30)</td>
<td>(n=16)</td>
<td>(n=33)</td>
<td>(n=15)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: ALA =aminolevulinic acid.

n = number of samples processed. p values >0.05 not shown; a−f p<0.01.
intensities of gastric pits in the antrum, corpus and fundus compared with the corresponding full-frame imaging fields are most likely due to the much lower level of fluorescence-generating capacity of the *H. pylori* in the pits as compared to the summation effect in the full-frame images. Both pit and full-field fluorescence values were higher with 405 nm excitation in *H. pylori* samples than in *H. pylori* samples regardless of location or treatment. The observed close correlation demonstrates the feasibility of FFI acquisition for clinical practice, where tissue sampling areas will be much larger and somewhat variable, and fully supports the concept that the information obtained from larger sampling areas correlates well with that obtained from pits alone and possesses comparable diagnostic accuracy.

Previous studies have successfully demonstrated the use of ALA-induced PPIX fluorescence for gastric *H. pylori* diagnosis using data from individual gastric pits and have shown, in agreement with the current study, that ALA-induced PPIX fluorescence (405 nm excitation) in *H. pylori* (+) is greater than in *H. pylori* (−) samples [14]. This distinction according to *H. pylori* status was more marked in the antrum than either the corpus or fundus, presumably because of the greater density of *H. pylori* in the antrum. In addition to the endogenous and ALA-induced PPIX fluorescence produced by *H. pylori* itself, it is possible that a fraction of the fluorescence seen with 405 nm excitation is attributable to the response of the gastric tissue to the *H. pylori* infection and the associated inflammation and increased cell proliferation in the gastric pits [6,7,20–22]. Both inflammation and cell proliferation have been correlated with an increased rate and amount of PPIX synthesis from endogenous and exogenous precursors [23–25]. Further studies should compare fluorescence in *H. pylori*- and non-*H. pylori*-associated gastritis. The small increase in ALA-induced fluorescence seen in the FFI *H. pylori* (−) subjects with ALA treatment is most likely due to the normal ability of the gastric mucosa to metabolize exogenous porphyrin precursors when additional ALA is made available [26–28].

Fluorescence at 535 nm excitation both with and without ALA did not distinguish between *H. pylori* (+) and *H. pylori* (−) subjects in the antrum. In the corpus and fundus, fluorescence at 535 nm was only distinctive without the use of ALA, where samples from *H. pylori* (−) subjects showed increased fluorescence compared with *H. pylori* (+) samples. Consequently, we speculate that
endogenous fluorescence of *H. pylori* at this wavelength of excitation is minimal despite 535 nm being a small absorption peak for *H. pylori* [18]. The slight increase in autofluorescence seen in the *H. pylori* (+) subjects after ALA treatment is most probably due to the weak absorption of the newly generated PPIX near the 535 nm excitation [15].

Ratios of different wavelengths of fluorescence emission to a single excitation wavelength can produce unique signatures for normal versus malignant tissue [29–31]. We examined the inverse situation, the ratio of total fluorescence emitted above a cut-off wavelength produced by excitation with different wavelengths, to determine whether

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Figure 6. Distribution of the ratios of fluorescence for 535 nm:405 nm excitation for 4 *H. pylori* (−) and 8 *H. pylori* (+) subjects with and without aminolevulinic acid (ALA) treatment. A. and B. Antrum. C. and D. Corpus. E. and F. Fundus.
Table III. Sensitivity and specificity for distinguishing *H. pylori* status at selected 535 nm:405 nm excitation ratio cut-off points.

<table>
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<th>Antrum</th>
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<tbody>
<tr>
<td></td>
<td>ALA(−)</td>
<td>ALA(+)</td>
<td>ALA(−)</td>
<td>ALA(+)</td>
<td>ALA(−)</td>
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<tr>
<td></td>
<td>H. pylori(−)</td>
<td>H. pylori(+)</td>
<td>H. pylori(−)</td>
<td>H. pylori(+)</td>
<td>H. pylori(−)</td>
<td>H. pylori(+)</td>
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<tr>
<td>Number</td>
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<td>22</td>
<td>57</td>
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<td>21</td>
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<td>57</td>
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<td>65</td>
<td>1.55−3.73</td>
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<td>0.77−2.68</td>
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<td>1.27−3.38</td>
<td>1.55−3.73</td>
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<tr>
<td>Cut-off</td>
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<td>≤1.58−H. pylori(+)</td>
<td>≤1.96−H. pylori(+)</td>
<td>≤2.03−H. pylori(+)</td>
<td>≤1.92−H. pylori(+)</td>
<td>≤1.78−H. pylori(+)</td>
<td>≤1.92−H. pylori(+)</td>
<td>≤1.78−H. pylori(+)</td>
<td>≤1.92−H. pylori(+)</td>
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<td>≤1.92−H. pylori(+)</td>
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<tr>
<td>Sensitivity</td>
<td>74.51%</td>
<td>78.95%</td>
<td>61.50%</td>
<td>66.67%</td>
<td>69.57%</td>
<td>67.9%</td>
<td>74.51%</td>
<td>78.95%</td>
<td>61.50%</td>
<td>66.67%</td>
<td>69.57%</td>
<td>67.9%</td>
<td>74.51%</td>
<td>78.95%</td>
<td>61.50%</td>
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<tr>
<td>Specificity</td>
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<td>61.50%</td>
<td>72.50%</td>
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<td>67.9%</td>
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<td>61.50%</td>
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Abbreviation: ALA = aminolevulinic acid.
laser fiber technologies required to do so are readily available and methodologies using white-light illumination have been reported [34–36]. There has also been a case report of a related endoscopic approach using confocal microscope technology to visualize individual *H. pylori* infection within the gastric pits using topically administered acriflavin hydrochloride or intravenously delivered fluorescein sodium [37].

**Conclusions**

Full-field fluorescence measurements correlate strongly with fluorescence in individual pits and provide accurate information on the presence and localization of gastric *H. pylori* infection. Topical application of ALA combined with 405 nm excitation improves *H. pylori* infection detection in all areas of the gastric mucosa. However, even without ALA treatment, it may be possible to detect *H. pylori* in the antrum using 405 nm excitation. Enhanced detection of *H. pylori* infection throughout the gastric mucosa based on the ratio of fluorescence emission with 535 nm excitation to that from 405 nm excitation may also be possible to achieve without further fluorescence enhancement by ALA treatment.

**Acknowledgements**

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