Chapter V

New immunoassay for the detection of Helicobacter pylori infection compared with urease test, 13C breath test and histology: validation in the primary care setting

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Abstract

*Helicobacter pylori (H pylori)* plays a major role in peptic ulcer disease and as a result testing for *H pylori* infection in patients with dyspepsia has often been advocated. Aim of the study was to determine the diagnostic accuracy, the analytical performance, and optimal cut off point of a new serological assay, the Pyloriset® EIA-G III for the detection of *H pylori* infection in the primary care setting. For 113 primary care patients with dyspepsia urea breath test, CLO™test, histology and serology tests were performed. Diagnostic accuracy of the Pyloriset® EIA-G III was evaluated against a reference standard of a carbon urea breath test (CUBT), CLO™ test and histology (from gastric biopsies). Precision, linearity and correlation of the serological assay with the CUBT and former Pyloriset® were also determined. At the optimal cut-off level of 40 U/ml the positive predictive value was 92.1%, negative predictive value 96.3%, sensitivity 87.5%, and specificity 93.9%. The within-run precision was high. The recovery data were good. The correlation of both CUBT and the former Pyloriset® EIA-G and the Pyloriset® EIA-G III was high. At the cut-off level of 40 U/ml the new Pyloriset® EIG-G III is a reliable method to detect *H pylori* infection in the primary care setting.
Introduction

The bacterium Helicobacter pylori (H pylori) is known to play a major role in the development of peptic ulcer disease. Infection with this spiral, urease producing bacterium causes histological gastritis and is an important risk factor for the development of gastric adenocarcinoma and lymphoma.\textsuperscript{1-4} Screening the whole general population for infection with H pylori does not appear to be cost-effective, but case finding in certain risk groups, i.e. patients with active ulcers, a history of ulcers, or gastric mucosa-associated lymphoid tissue lymphoma is indicated.\textsuperscript{5,6}

Many invasive and non-invasive methods are available for the detection of H pylori infection. Invasive methods require an endoscopy to obtain biopsies of gastric tissue, in which H pylori can be diagnosed by urease activity, histology or culture of the bacterium. Endoscopy is an inconvenient and expensive method of H pylori testing (approximately EURO 250 per endoscopy including invasive H pylori testing) and reliable non-invasive methods could be of great help, notably in the primary care setting. Non-invasive techniques to detect bacterial infection include carbon urea breath tests (CUBT), antigen stool tests and anti-H pylori antibody detection by serological methods.\textsuperscript{7,8} The antigen stool test is promising but needs further validation in different patient settings. The $^{14}$C-UBT is a simple and reliable test, but the radioactive component restricts its practical use. The $^{13}$C-UBT does not have the disadvantage of radioactivity, but requires the availability of an expensive mass spectrometer, resulting in a total cost of EURO 45 per $^{13}$C-UBT (personnel, equipment and materials). The test characteristics of most office tests, such as whole blood tests, are disappointing.\textsuperscript{9,10}

A fast and reliable test method to diagnose H pylori infection is to test for antibodies to the antigen of H pylori. The enzyme immunoassay is the most commonly used serological test, because it is a reliable, fast and low cost technique (per test approximately EURO 1 for personnel, equipment and materials). Many serological kits for the detection of H pylori-specific IgG antibodies are now commercially available.\textsuperscript{11,12} Aim of this study was to investigate the analytical performance and reliability of a new serological assay, the Pyloriset\textsuperscript{®} EIA-G III, for the detection of H pylori infection in the primary care setting. We investigated its test characteristics and analytical performance against a reference standard of CUBT and invasive tests, and against the former Pyloriset,\textsuperscript{®} and determined the optimal cut off level for the new test.
Methods

Patients
Patients were recruited from general practices in the city of Utrecht, the Netherlands. Eligible for the study were patients that presented themselves to their GP with dyspepsia lasting at least two weeks and were ≥18 years old. Pregnant patients and patients with pulmonal or cardiac comorbidity were excluded. The patients were referred to the local primary care laboratory for serological screening for H pylori infection (Pyloriset® EIA-G and Pyloriset® EIA-G III, Orion Diagnostica, Espoo, Finland). At the same visit the patients also underwent a 13C urea breath test (Pylobactell™, BSIA/Torbet laboratories, Chatham, United Kingdom); the breathtest samples were analysed and results expressed as 13CO2/12CO2 concentrations (an increase in 13CO2/12CO2, concentrations from baseline of more than 3.5 ‰ was required for an established H pylori infection). Subsequently, patients were referred to the local hospital for endoscopy, during which biopsy samples were taken for histology (using Giemsa or Haematoxylin/Eosin stain), and a rapid urease test (CLO™, Australia).

Diagnosis with Pyloriset® EIA-G III
The newly developed EIA kit, Pyloriset® EIA-G III assay uses microtiter wells coated with inactive H pylori antigens. In the present study, the assay procedure steps were automated using the Biolab 300 (Meridian diagnostics, Cincinnati, USA). Serum samples were diluted (1:201) with serum dilution buffer. Four undiluted calibration sera and diluted samples were added to the wells, mixed, and incubated at 25°C for 30 minutes. The plate was washed three times with washing buffer, and then conjugate (peroxidase conjugated anti-human IgG (rabbit)) was added to each well. After mixing and a second incubation at 25°C for 30 minutes, the plate was washed again. Substrate (3,3',5,5'-tetramethylbenzidine) was then added to each well and the plate mixed and incubated at 25°C for 10 minutes. The reaction was stopped by adding the stopping solution (1M H2SO4) and the absorbance of the assay was read at 450 nm. The optical densities of four reference standards were used to plot a standard curve (straight line) by which the H pylori IgG antibody levels in patient samples were quantified. The results were expressed in arbitrary units per milliliter. Reference standards 1 to 4 represent 10, 20, 120, 640 U/ml, respectively. The absorbance readings are proportional to the logarithm of the antibody concentration. Following the manufacturer’s interpretation of the assay the result should be considered positive for H pylori antibodies if the U/ml of the serum is equal or higher than that of the calibrator serum 2 (≥20
U/ml). To compare the new with the former *H pylori* assay, the arbitrary units were multiplied with a factor 10 (recommended by the manufacturer) to reset these values in titer values as used in the former Pyloriset® EIA-G new kit. The total procedure time for the Pyloriset® EIA-G III was 80 minutes.

**Definition of the reference standard**
Patients were considered to be *H pylori* infected if at least two of the following three tests were positive: 1) rapid urease test (CLO™), 2) ¹³C urea breath test (CUBT), 3) histology.

**Analytical performance of the Pyloriset® EIA-G III**

**Precision**
Within-run precision was determined using sera on three levels. Replicate measurements (n=20) were performed in one run for each level. This procedure was processed using a single reagent lot. The within-run precision data are expressed as coefficients of variation (CV, %). Between-run precision was determined using sera on three levels. Replicate measurements (n=5) were performed in different runs. A single reagent lot was used during the measurements. The between-run data were expressed as CV’s.

**Linearity**
The linearity of the Pyloriset® EIA-G III was assessed by calculation of the recovery of a repeatedly diluted high concentrate sample (~800 U/ml).

**Effect of re-thawing sera on detection with pyloriset® EIA-G III**
The possible effect of re-thawing sera on the IgG antibody levels was tested with sera at 3 levels. The sera have been thawed and frozen again for 5 times and analyzed. The possible effect of re-thawing is expressed as CV.

**Correlation of tests**
Correlations between the qualitative test results (i.e. positive or negative) of the Pyloriset® EIA-G III with the CUBT, and Pyloriset® EIA-G new were determined using Cohen’s kappa (measurement of agreement).

**Statistical analysis**
The test characteristics were reported in terms of sensitivity, specificity, positive and negative predictive values. Measurement of agreement was reported in terms of Cohen’s kappa. Statistical analysis was performed using SPSS version 9.0 for Windows.
Table 1  Characteristics of the Pyloriset® EIA-G III at different cut-off levels in relation to the reference standard (95% confidence interval)

<table>
<thead>
<tr>
<th>Cut-off level</th>
<th>20</th>
<th>30</th>
<th>35</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPV</td>
<td>69.8 (55.7-81.7)</td>
<td>78.7 (64.3-89.3)</td>
<td>83.7 (69.3-93.2)</td>
<td>92.1 (78.6-98.3)</td>
</tr>
<tr>
<td>NPV</td>
<td>98.5 (92-100.0)</td>
<td>98.6 (92.6-100.0)</td>
<td>97.4 (90.9-99.7)</td>
<td>96.3 (89.4-99.2)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>97.4 (86.2-99.9)</td>
<td>97.4 (86.2-99.9)</td>
<td>94.7 (82.2-99.4)</td>
<td>87.5 (73.2-95.8)</td>
</tr>
<tr>
<td>Specificity</td>
<td>80.5 (70.3-88.4)</td>
<td>87.8 (78.7-94.0)</td>
<td>91.5 (83.2-96.5)</td>
<td>93.9 (86.3-98.0)</td>
</tr>
</tbody>
</table>

PPV: positive predictive value, NPV: negative predictive value

Results

Patients
Between April 1999 and January 2000, 133 primary care patients with dyspepsia were included, and referred for *H pylori* testing and endoscopical diagnosis. For 113 patients complete data on both the EIA and the reference standard were available. The *H pylori* infection rate, according to the reference test (consisting of CUBT, CLO and histology), was 31.7%.

Validation against reference standard
The observed negative and positive predictive values, sensitivities and specificities of the Pyloriset® EIA-G III assay are given in table 1. Test characteristics were determined at different cut-off levels (above which an individual was considered to be *H pylori* infected). At cut-off levels for infection varying from 20-40 U/ml, positive predictive values ranged from 69.8-92.1%, negative predictive values from 96.3-98.6%, sensitivities from 87.5-97.4%, and specificities from 80.7-93.3%.

Table 2  Table demonstrating the results of dilution in terms of recovery using the Pyloriset® EIA G III

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Value (U/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>806</td>
<td>100</td>
</tr>
<tr>
<td>A/2</td>
<td>360</td>
<td>89</td>
</tr>
<tr>
<td>A/4</td>
<td>179</td>
<td>99</td>
</tr>
<tr>
<td>A/8</td>
<td>75</td>
<td>84</td>
</tr>
</tbody>
</table>
Table 3  Percentage of patients incorrectly diagnosed in a population with \textit{H pylori} infection rate of 40\% at different cut-off levels

<table>
<thead>
<tr>
<th>Cut-off level</th>
<th>20</th>
<th>30</th>
<th>35</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>False positives</td>
<td>9</td>
<td>6</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>False negatives</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Analytical performance of the Pyloriset® EIA-G III

\textit{Precision}

The within-run precision data expressed as CV’s of the mean levels of 10 U/ml, 133 U/ml and 503 U/ml were respectively 1.6, 8.4 and 11.2\%. At the mean levels of 11 U/ml, 93 U/ml and 614 U/ml, the between-run precision CV’s were 4.8, 7.5 and 28.7\% respectively.

\textit{Linearity}

The calculated recovery data are shown in table 2. Good linearity data were observed, with recovery data above 80\% for all the 2, 4 and 8 times dilutions.

\textit{Effect of re-thawing sera on detection with Pyloriset® EIA-G III}

The CV’s measured for the samples at mean levels of 10.5, 162 and 892 U/ml, that had been thawed and frozen again, were respectively 3.4, 13.5 and 28.4 \%.

\textit{Correlation of tests}

The correlation (comparing qualitative test results) between the breath test and Pyloriset® EIA-G III was high: Cohen’s kappa of 0.92. This result was similar for cut-off levels of 35 or 40 IU/ml for the Pyloriset® EIA-G III as level of proven infection. The correlation between the former Pyloriset® EIA-G and the Pyloriset® EIA-G III was high; Cohen’s Kappa of 0.97 or 0.98 (using cut-off values of 40 U/ml or 35 U/ml respectively).

Discussion

The new Pyloriset® is a reliable method to detect \textit{H pylori} infection in primary care. Test characteristics validated against a high quality reference standard are excellent, and correlation of results with those of CUBT and the former EIA is good. The recommended cut-off value needs to be carefully reconsidered. The
The cut-off value of the former Pyloriset® assay as given by the manufacturer was a titer of 300. Surprisingly for the Pyloriset® EIA-G III assay, a cut-off value of 20 U/ml is recommended by the manufacturer, where a value of 30 U/ml was expected according to the factor 10 difference between the two assay’s. In clinical practice, supporting clinical decisions for individual patients, the optimal cut-off point should be guided mainly by the positive and negative predicted value of the test (the PPV and NPV). The fact that \textit{H pylori} diagnosis will be mainly used in dyspepsia management to support ulcer detecting strategies (in particular endoscopy plus antibiotic treatment), puts even more emphasis on the need for a correct \textit{H pylori} test result, both positive and negative. Calculating the percentage of incorrectly diagnosed or missed \textit{H pylori} infections at different cut-off levels demonstrates the most efficient cut-off point in clinical dyspepsia management in primary care (table 3). At 40 U/ml only 5% of the patients are incorrectly diagnosed.

Validation of the former assay (Pyloriset® EIA-G New) in a similar primary care population resulted in a sensitivity of 91% and specificity of 78%.\textsuperscript{13} We used a more solid reference standard (at least 2 out of 3 reference tests positive, versus only one reference test used by Lewin-van den Broek), which resulted in a sensitivity of 94.7% and specificity of 92.7%. The new assay (EIA-G III) performs in a similar way in terms of sensitivity, specificity, positive and negative predictive value as the former EIA-G New.

A few additional advantages of the new EIA should be addressed. The total procedure time of the EIA-G III is 80 minutes vs. 160 minutes in the EIA-G New. In contrast to the former EIA, the EIA-G III has a straight calibration line, which leads to accurate results in the whole calibration range. No disadvantages in comparison with the former Pyloriset® could be found.

In conclusion this new serology assay is an accurate, reliable and inexpensive screening test for \textit{H pylori} infections in dyspeptic patients in a primary care population, but the cut-off level for use in primary care should be increased from 20 to 40 U/ml.

\textbf{Acknowledgements}

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References