Correlation between serum levels of vitamin B\textsubscript{12} and anti-\textit{Helicobacter pylori} IgA antibodies in vitamin B\textsubscript{12} deficient Palestinian patients

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1. Introduction
\textit{Helicobacter pylori} infections induce vigorous systemic and mucosal humoral responses that are predominantly mediated by IgA, IgG, and IgM. These immunoglobulins are detectable in sera, gastric aspirates, or stomach extracts (1,2). Humoral immunity against \textit{H. pylori} can effectively prevent infection and reduce colonization but does not lead to eradication of \textit{H. pylori}-induced gastritis (2).

Absorption of dietary cobalamin (vitamin B\textsubscript{12}, vB\textsubscript{12}) depends on several factors, including acid-dependent deproteinization of vB\textsubscript{12}. Only free vB12 can form a complex with the intrinsic factor (IF). The vB12–IF complex is then absorbed by mucosal cells via its specific receptor (cubilin) in a calcium-dependent fashion. Gastric parietal cells are responsible for production of both hydrochloric acid and IF. Absorbed vB12 is then stored in the liver.

Some \textit{H. pylori} patients develop autoantibodies directed against gastric parietal H\textsuperscript{+}/K\textsuperscript{–}-ATPase cells (APCAs), resulting in achlorhydria and increased infection with \textit{H. pylori}, which in turn contributes to gastric damage and atrophy of the corpus (2).

2. Methods and materials
2.1. Patients
Blood samples were collected in 5 mL tubes from 133 vB\textsubscript{12}− deficient patients (60 males, 73 females) and 105 healthy volunteers (42 males, 63 females), aged 18–50 years (mean, 34.1 years). All 133 patients had vB\textsubscript{12} deficiency and

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Helicobacter pylori dyspeptic symptoms such as epigastric pain, nausea, heartburn, and vomiting (7, 8).

Informed consent was obtained from each participant (patients and healthy volunteers). Interviews and questionnaires were administered in Arabic to all participants. Sera were collected starting November 2009 through February 2010.

Collected data regarding smoking habits, age, sex, symptoms, and causes of \( vB_{12} \) deficiency were recorded for each participant. A healthy volunteer was defined as one who did not suffer from disorders or stomach problems, i.e. absence of gastritis.

2.2. Subject exclusion criteria

In order to focus our research on the relationship between Helicobacter pylori infection and \( vB_{12} \) deficiency, factors and cases known to affect the state of \( vB_{12} \) levels were excluded, with the exception of \( H. pylori \) infection. Subjects suffering from known causes of \( vB_{12} \) deficiency other than \( H. pylori \) infection were excluded and patients suffering from other chronic diseases that may interfere with the interpretation of the results of this study were excluded as well.

The exclusion criteria were as follows: age <18 or >50 years; diabetic patients; celiac disease; patients with a history of (steroid or nonsteroid) antiinflammatory drug, antacid, \( H_{2} \)-receptor antagonist, or proton pump inhibitor intake in the previous month; history of treatment of \( H. pylori \) infection; history of drug \( vB_{12} \) intake; patients with gastrointestinal bleeding, renal failure, liver diseases, thyroid disorders, autoimmune diseases, previous GI surgery, folate deficiency, strict vegetarian, neoplasia, alcohol intake, rheumatoid arthritis, or pregnancy.

2.2.1. Exclusion of \( vB_{12} \)-deficient patients showing anti-\( IF \) activity

The presence of anti-\( IF \) antibodies in sera of \( vB_{12} \) deficient patients was determined using an IF ELISA kit (Alpha S.A NV, Belgium), following the manufacturer’s instructions. Samples were diluted to 1:51, mixed, and 100 \( \mu L \) of each sample dispensed into a designated microwell. After 30 min of incubation at room temperature, microwells were washed with diluted washing buffer (1:20) and treated with 100 \( \mu L \) of conjugate for 30 min. Afterwards, substrate (100 \( \mu L \)) was added and incubated for 10 min at room temperature. After adding the stop solution, absorbance was recorded at 450 nm. A sample was considered negative when the binding index (BI) was less than 1.0; a sample was considered positive when BI was >1.0. All positive subjects were excluded from the study.

2.2.2. Exclusion of subjects showing APCAs

APCAs were determined in sera with a commercial enzyme immunoassay (Varelisa Parietal Cell Antibodies, Pharmacia Diagnostics, Germany). All sera samples with \( vB_{12} \) deficiency (diluted 1:101) were processed according to manufacturer’s instructions and absorbance was read at 450 nm within 30 min of adding stop solution in reference to absorbance recorded at 620 nm. A sample was scored as negative when the ratio was <1.0, a sample was scored as equivocal when the ratio ranged between 1.0 and 1.4, and a sample was positive when the ratio was >1.4. All positive subjects were excluded from the study.

The decision to exclude these subjects (Sections 2.2.1 and 2.2.2) was based on published literature showing the presence of APCAs among children (9) and diabetic adults (10) who do not have \( H. pylori \) infections. Exclusion was necessary to have a clear view of the relationship between \( H. pylori \) and \( vB_{12} \) deficiency in the absence of any potential contaminating factor (including autoimmunity to parietal cells or IF), and that the results were a true reflection of the direct relationship of \( vB_{12} \) deficiency to \( H. pylori \) infection.

2.3. \( vB_{12} \) deficiency

\( vB_{12} \) was quantified in sera using AxSYM Abbott automation system (Abbott Laboratories, USA), a method based on microparticle–enzyme–IF assay; the results were expressed in pg/mL. The cutoff level for \( vB_{12} \) deficiency is <200 pg/mL, according to the manufacturer’s instructions.

The following scale was adopted to estimate and define \( vB_{12} \) level of deficiency among patients: severe deficiency when the mean \( vB_{12} \) value is <75 pg/mL of serum, moderate deficiency when the mean \( vB_{12} \) value is between 75 and 150 pg/mL, and mild deficiency when the mean \( vB_{12} \) ranges from 150 to <200 pg/mL (200 pg/mL being the cutoff value between deficiency and sufficiency). Normal \( vB_{12} \) range is >200 to 900 pg/mL. (11).

2.4. Quantification of serum anti-Helicobacter pylori IgA

Anti-Helicobacter pylori IgA concentration was determined in sera samples using an \( H. pylori \) IgA ELISA kit (NovaTec Immunodiagnostica; GmbH, Germany) as instructed by the manufacturer. Microwell plates and reagents were brought to room temperature (25 °C). Washing buffer (diluted 1:19 in distilled water) and serum samples (10 \( \mu L \)) were mixed with 0.99 mL IgA diluents. Then 100 \( \mu L \) of each standard (A, B, C, D) and diluted samples were placed into their designated wells, covered, and incubated at 37 °C for 60 min. Microwells were washed 3 times with 300 \( \mu L \) diluted washing buffer. Except for blank wells, 100 \( \mu L \) of Helicobacter pylori anti-IgA conjugate were added to each well. Plates were incubated in the dark for 30 min at room temperature. This was followed by another cycle of washing as above. Substrate (100 \( \mu L \)) was added to each well (including the blanks), covered, and incubated for 15 min at room temperature (25 °C) in the dark. Stop solution (100 \( \mu L \)) was added to each well (including the blanks), covered, and incubated for 15 min at room temperature in the dark. The blue/orange color of wells was scored for each well using photometric measurements at 450/620 nm within 30 min. The scale recommended by manufacturer was adopted. \( H. pylori \) infection was considered reactive if the mean serum IgA value was >20 NTU/mL, equivocal
if the mean serum IgA titer fell between 15 and 20 NTU/mL, and nonreactive if the mean serum IgA value was <15 NTU/mL (healthy or immune-tolerant).

2.5. Statistical analysis and graphs

Collected data were analyzed using Microsoft Excel (2007) and the online Social Science Statistics (http://www.socscistatistics.com/tests/mannwhitney/Default.aspx) (10). Analyses and calculations included means, standard deviation, median, upper and lower limits, Mann–Whitney significant differences between control and patient groups at \( P < 0.05 \) and \( P < 0.01 \), and open form \( P \)-values.

Correlation analyses were based on Excel Pearson’s \( R^2 \) and its square root \( (r) \), while confidence was determined using the two-tailed “r” distribution at \( P < 0.05 \) and \( P < 0.01 \) and open form \( P \)-values. Additionally, Pearson’s correlation levels were analyzed after removing potential contaminating variables (factors) which included subjects with abnormal vB_{12} or abnormal IgA titers and some outlier values as described in Section 3.

3. Results

3.1. Levels of vB_{12} and IgA

Levels of vB_{12} and anti-\textit{Helicobacter pylori} IgA in sera are presented in Figure 1 and the Table. Means and medians for each of the four groups of subjects were obtained. The median of serum vB_{12} level for patients (150; range 59–198 pg/mL serum) was significantly different from the median for the control group (330; range 187–731 pg/mL serum, \( P = 0.00001 \)) and the medians were significantly different at \( P < 0.05 \) and \( P < 0.01 \) (Mann–Whitney U-test) (Figure 1 and Table).

Significant differences (\( P < 0.05 \) and \( P < 0.01 \); \( P = 0.00001 \)) were recorded for IgA levels, indicating that the median IgA for patients (40; range 6–153 NTU/mL serum) was different from the median for control subjects (range 0–140 NTU/mL serum) (6). Severe vB_{12} deficiency was observed in 4.5% (6/133 patients); moderate vB_{12} deficiency in 48.9% (65/133). The remaining 62 patients (46.6%), showed mild vB_{12} deficiency. Healthy control subjects had normal vB_{12} levels, ranging from 212 to 756 pg/mL serum with the exception of one subject, a 35-year-old female that showed vB_{12} deficiency (187 pg/mL) and high IgA titer (22 NTU/mL).

The majority of healthy participants (85.7%, 90/105) had background levels of anti-\textit{Helicobacter pylori} IgA (0 to 14 NTU/mL). Fifteen control subjects (14.3%; 15/105 including the 35-year-old female with vB_{12} deficiency) had IgA titers higher than 15 NTU/mL, ranging from 22 to 140 NTU/mL serum. The vast majority of vB_{12}-deficient patients (84.2%; 112/133) had IgA titers higher than 20 NTU/mL, while the remaining patients (15.8%; 21/133) had low levels of IgA (Table). The two groups (15 control and 22 patient subjects) were viewed as contaminating factors (Table and Section 3.2.). There was no correlation between age and vB_{12} deficiency (\( r = -0.0495; P = 0.44 \)) nor between age and IgA titers (\( r = 0.0207; P = 0.679 \)).

3.2. Correlation between vB_{12} levels and anti-\textit{H. pylori} IgA

Pearson’s correlation coefficient ‘r’ between serum vB_{12} and anti-\textit{H. pylori} IgA levels was \(-0.4809; P = 0.00001 \) as determined for all 238 participants (105 controls and 133 patients). However, when 15 control subjects (showing IgA

\[ \text{(Figure 1). Vitamin B}_{12} (\text{vB}_{12}; \text{pg/mL}) \text{ and anti-}\text{H. pylori} \text{ IgA (IgA; NTU/mL) serum levels. Mean ± standard deviation (SD) among control (vB12-C) or patients (vB12-P) subjects are presented as shaded bars. Median values for each category are presented as clear bars. The corresponding lower and upper limits are given in the inset table. Mann–Whitney test was significant (P < 0.05 and P < 0.01; P = 0.00001) for vB12 or IgA relative to their control values; see Table.} \]
titers >15 NTU/mL) were excluded, a stronger correlation coefficient was obtained (r = –0.539; P = 0.00001). Upon the omission of 13 patient subjects showing IgA titers <15 NTU/mL, the obtained correlation coefficient was r = –0.521; P = 0.00001. When both contaminating groups (15 controls and 13 patients) were omitted, the correlation became stronger (r = –0.579; P = 0.00001). A further omission of a group of controls (7 subjects) showing high vB12 concentration (>537 pg/mL serum) a further increase in correlation was obtained (r = –0.615; P = 0.00001). The results showed significant Pearson’s correlation both at P < 0.05 and P < 0.01.

4. Discussion

4.1. vB12 stores

The liver is the main vB12 store; it stores 80% of total body vB12 (2 to 5 mg). Stored vB12 will last an adult individual for 3–5 years in the absence of significant vB12 intake, or for 5–6 years when vB12 intake is insufficient. In addition, vB12 undergoes a daily enterohepatic circulation where 1 to 10 µg is excreted in bile and reabsorbed (6). Accordingly, it is expected that only chronic H. pylori infections, not recently acquired infections, will be associated with H. pylori-induced vB12 deficiencies. Such a correlation was demonstrated in this study. The presented results confirmed that IgA titers were high in 15 control subjects (14.3%); one of them (a 35-year-old female) showed vB12 deficiency, indicating that H. pylori infection preceded vB12 deficiency and suggesting that H. pylori infection may indeed have contributed to vB12 deficiency or caused it. The results suggest the existence of another mechanism for the role of H. pylori in vB12 deficiency other than APCAs or anti-IF antibodies. Although accumulating evidence suggests that H. pylori infection has a negative effect on the absorption of vB12 (12), the inhibition of vB12 absorption by H. pylori infection cannot yet be verified.

4.2. Identification of contaminating variables that influenced correlation analysis

This study identified and excluded at least five contaminating factors; these factors would have blurred the correlation

<table>
<thead>
<tr>
<th>Data exclusion</th>
<th>Subject group</th>
<th>Sample size (n)</th>
<th>Median</th>
<th>Lower–upper limits</th>
<th>Mean</th>
<th>SD/SEM</th>
<th>Pearson’s correlation (r)</th>
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</thead>
<tbody>
<tr>
<td>• Raw data</td>
<td>vB12-C</td>
<td>105</td>
<td>330*</td>
<td>187–731</td>
<td>357.5</td>
<td>97.5</td>
<td>–0.45*</td>
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<tr>
<td></td>
<td>IgA-C</td>
<td>105</td>
<td>6</td>
<td>0–140</td>
<td>13.6</td>
<td>23.1</td>
<td>Or –0.538*</td>
</tr>
<tr>
<td></td>
<td>vB12-P</td>
<td>133</td>
<td>150*</td>
<td>59–198</td>
<td>151.5</td>
<td>31.6</td>
<td>Or –0.521*</td>
</tr>
<tr>
<td></td>
<td>IgA-P</td>
<td>133</td>
<td>40</td>
<td>6–153</td>
<td>59</td>
<td>45.7</td>
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</tr>
<tr>
<td>• Removed 15C or 13P</td>
<td>vB12-C</td>
<td>105/90</td>
<td>335</td>
<td>212–665</td>
<td>362</td>
<td>97.5/92.6</td>
<td>Or –0.538*</td>
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<tr>
<td></td>
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<td>105/90</td>
<td>5</td>
<td>0–14</td>
<td>6</td>
<td>23.1/3.9</td>
<td>Or –0.521*</td>
</tr>
<tr>
<td></td>
<td>vB12-P</td>
<td>133/120</td>
<td>150</td>
<td>59–198</td>
<td>152/152</td>
<td>31.6/31.1</td>
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<tr>
<td></td>
<td>IgA-P</td>
<td>133/120</td>
<td>40</td>
<td>0–153</td>
<td>59/63</td>
<td>45.7/45</td>
<td></td>
</tr>
<tr>
<td>• Removed 15C and 13P (28 subjects)</td>
<td>vB12-C</td>
<td>105/90</td>
<td>335</td>
<td>212–665</td>
<td>362</td>
<td>97.5/92.6</td>
<td>–0.58*</td>
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<tr>
<td></td>
<td>IgA-C</td>
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<td>0–14</td>
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<tr>
<td></td>
<td>IgA-P</td>
<td>133/120</td>
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<td>0–153</td>
<td>59/63</td>
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<tr>
<td>• Removed 15C + 13P + 7C</td>
<td>vB12-C</td>
<td>83</td>
<td>326.5</td>
<td>212–665</td>
<td>379</td>
<td>62.8</td>
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<tr>
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<td>IgA-C</td>
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<td>0–14</td>
<td>6</td>
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<td>vB12-P</td>
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<td>59–198</td>
<td>161</td>
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<tr>
<td></td>
<td>IgA-P</td>
<td>120</td>
<td>40</td>
<td>0–153</td>
<td>58</td>
<td>32.2</td>
<td></td>
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</table>

*Significant correlation at P < 0.05 and P < 0.01; P = 0.00001.

Data exclusion category: exclusion of 15 (15C) control subjects showing IgA titers >15 NTU/mL and/or 13 patients (13P) with IgA titers <15 NTU/mL. Exclusion of seven additional control (7C) subjects (4th group) with vB12 values (537–665 pg/mL).

Φ, ¶ Mann–Whitney differences at P < 0.05 and P < 0.01; P = 0.00001.
between vB\textsubscript{12} deficiency and \textit{H. pylori}. Subjects with anti-
IF activity or APCAs were therefore excluded, since vB\textsubscript{12} in
these subjects cannot be directly correlated to \textit{H. pylori}
infection and would have acted as contaminating variables.
The study was further strengthened by excluding patients
consuming supplementary vB\textsubscript{12} or those diagnosed with
other diseases, such as celiac disease. Others were excluded
based on their responses to structured questionnaire and
interviews (see Section 2.2). This study has statistically
confirmed previous observations and suggestions (12–19)
linking vB\textsubscript{12} deficiency to \textit{H. pylori} infection. Kaptan et al.
(15) found \textit{H. pylori} in 77 (56\%) of vB\textsubscript{12}-deficient patients;
others predicted correlation or a cause–effect relationship
between vB\textsubscript{12} deficiency and \textit{H. pylori} infection, with
possible destruction of parietal cells as a result of \textit{H. pylori}
infection (10, 21). This study determined the actual
negative state of correlation between the two variables ($r = -0.45$; $P = 0.00001$). The correlation between \textit{H. pylori}
infections as indicated by serum levels of anti-\textit{H. pylori}
IgA and vB\textsubscript{12} deficiency was affected by contaminating
variables; a fraction of the control group (15/105; 14.3\%)
showed high IgA titers, suggesting possible asymptomatic
\textit{H. pylori} infection. Another contaminating factor appeared
among patient subjects in the form of IgA titers lower than
the cutoff value (<15 NTU/mL serum). The impact of
these factors on correlation was demonstrated when they
were excluded from data before analysis. The omission of
15 contaminating control subjects caused an increase in
correlation ($r = -0.538$). A similar contribution was made
upon omission of 13 patients showing IgA titers lower
than 15 NTU/mL ($r = -0.521$). When both contaminating
variables were omitted, a stronger correlation emerged
($r = -0.58$). Omission of seven (6.6\%) additional control
subjects showing high levels of vB\textsubscript{12} (536–731 pg/mL
serum; Figure 2A) increased the correlation coefficient
to $r = -0.61$.

4.3. Anomalies among subjects
Figure 2 illustrates that subjects can be categorized into
one of four clusters based on the combined level of vB\textsubscript{12}
and anti-\textit{H. pylori} IgA. Cluster A reflects normal healthy
subjects with high vB\textsubscript{12} (>200 pg/mL) and low IgA (<15
NTU/mL). Cluster B contains healthy controls with high
vB\textsubscript{12} but with elevated anti-\textit{H. pylori} IgA (>15 NTU/
ml), which may be indicative of asymptomatic infection
or recent exposure. Among patients with mild vB\textsubscript{12}
deficiency (>150 but <200 pg/mL), cluster C showed poor
IgA response, whereas cluster D subjects showed high or
exaggerated IgA titers (subcluster D2). Although we do not
have an explanation for these variations, several possible
explanations can be put forward for future considerations.
First, antigenic variations among \textit{H. pylori} variants may
render the anti-\textit{H. pylori} IgA determination kit insensitive
to some antigenic variants of \textit{H. pylori} (18,20–23), i.e. IgA
kit cannot equally detect all \textit{H. pylori} antigenic variants.
Second, these patients, or some of them, cannot mount

![Figure 2](image-url)

**Figure 2.** Scatter blot of all 238 subjects; vB\textsubscript{12} was blotted against its cognate IgA
value. The cutoff value for IgA (15 NTU/mL) ($y = 15$) and the cutoff value for vB\textsubscript{12}
($x = 200$ pg/mL) are indicated. Accordingly, several clusters/trends (A–D) can be
distinguished. **A:** High vB\textsubscript{12} and low IgA (typical normal healthy control subjects),
**B:** high vB\textsubscript{12} and high IgA (possible asymptomatic patients; a potential contaminating
factor, see Section 4), **C:** low vB\textsubscript{12} and low IgA (patients not responding to infection;
another potential contaminating factor), **D:** low vB\textsubscript{12} and moderate or high IgA
(majority of typical patients); **D1:** normal patients (IgA < 124), **D2:** a subset of
patients showing exaggerated IgA levels (>144 NTU/mL serum).
a significant IgA immune response against *H. pylori* (i.e. they are immune-tolerant). Third, vB12 deficiency among cluster C patients, or some of them, was the result of a factor independent of *H. pylori*. Exaggerated IgA levels observed in some patients (Figure 2, D2), may be due to repetitive exposure to living or dead *H. pylori* antigens (e.g., drinking *H. pylori*-contaminated or chlorinated well water). Another possibility resides in the potency of the subject's immune response to different *H. pylori* antigenic variants.

4.4. Incidence and baseline of asymptomatically infected population

A conclusion regarding the incidence and baseline of an asymptomatically infected population was calculated to be approximately 14.3% (15/105) as represented by the high titer (>15 NTU/mL serum) of anti-*H. pylori* IgA among normal control subjects aged 18 to 50 years from the Hebron area. In an earlier study by Serin et al. (24), 25% of healthy children were PCR-positive for *H. pylori* (24). Since some of the children were only transiently infected while others may have progressed to become symptomatic (25), it is likely that the percentage of asymptomatic subjects will decrease (i.e. <25%); accordingly it is likely that the baseline falls somewhere above 14.3% and below 25%.

In 9.8% of patients (13/133), IgA level did not predict the state of *H. pylori* infection (Figure 2C); IgA titers were below the cutoff value of the test (15 NTU/mL serum). Possible antigenic variants of *H. pylori* or other microbes may have caused gastritis and cannot be detected by the IgA test used in this study. Another possibility is that some subjects (including control subjects) were immune-tolerant to the *H. pylori*-targeted antigen. This category of IgA-negative subjects, in addition to false negative test results among all subjects, will shift the base line to a level higher than 14.3% when taken into consideration.

Additional studies across a given population are needed to obtain a realistic profile of the epidemiology of *H. pylori* infections.

4.5. Conclusions

Infection with *H. pylori* was negatively correlated to serum levels of vB12, and may contribute to this deficiency. If other categories (e.g., individuals aged <18 and >50) and factors are to be included in similar future studies, the baseline of asymptomatically infected healthy subjects may be significantly higher than 14.3%.

Serum level of anti-*H. pylori* IgA appears to be a good indicator of risk of developing vB12 deficiency. It is our recommendation that subjects showing high IgA titers should be treated for *H. pylori*, monitored, and prophylactically treated for vB12 deficiency. Failure of the IgA test to predict acute or chronic *H. pylori* infections in 9.8% of patients justifies the need for an additional supplementary or alternative test capable of detecting *H. pylori* in these and similar subjects, including apparently healthy subjects.

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