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Correlation between serum levels of vitamin B_{12} and anti-*Helicobacter pylori* IgA antibodies in vitamin B_{12} deficient Palestinian patients

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Background/aim: *H. pylori* infection and vitamin B_{12} (vB_{12}) deficiency have high prevalence rates among Palestinians. It was observed that most people who suffered from vB_{12} deficiency were positive for *H. pylori*.

Materials and methods: The correlation between *H. pylori* infection and vB_{12} deficiency was investigated in a representative segment of the Palestinian population. ELISA was used to determine levels of vitamin B_{12} (vB_{12}) and anti-*H. pylori* IgA in sera from 238 participants from Al-Khalil district (Hebron), Palestine.

Results: There was a strong negative Pearson's correlation coefficient ($r = -0.45$; $P = 0.00001$) between levels of anti-*H. pylori* IgA and vB_{12} levels in sera drawn from 238 participants (133 patients and 105 control subjects). Two important contaminating variables were identified in this study: healthy control subjects with elevated anti-*H. pylori* IgA titers and vB_{12}-deficient patients testing negative for anti-*H. pylori* IgA antibodies. The exclusion of the sources of contamination resulted in a stronger negative correlation; $r = -0.58$ ($P = 0.00001$).

Conclusion: The study provided a good screening system that may predict vB_{12} deficiency before its actual manifestation. If not treated, asymptomatic subjects showing increased anti-*H.pylori* IgA titers (>15 NTU/mL) are likely to be at risk of developing vB_{12} deficiency.

Key words: *Helicobacter pylori*, vitamin B_{12} deficiency, cobalamin, gastroenteritis, correlation, baseline

1. Introduction

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 *H. pylori* can effectively prevent infection and reduce colonization but does not lead to eradication of *H. pylori*-induced gastritis (2).

Absorption of dietary cobalamin (vitamin B_{12}; vB_{12}) depends on several factors, including acid-dependent deproteinization of vB_{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 *H. pylori* patients develop autoantibodies directed against gastric parietal H^+/K^+-ATPase cells (APCAs), resulting in achlorhydria and increased infection with *H. pylori,* which in turn contributes to gastric damage and atrophy of the corpus (2). Autoimmunity may destroy parietal cells that are responsible for hydrochloric acid and IF production, resulting in achlorhydria and failure to deproteinize vB_{12} and/or failure to produce IF. Consequently, the vB_{12}–IF complex fails to form, leading to malabsorption of vB_{12} and, potentially, to pernicious anemia. Hence, patients diagnosed with vB_{12} deficiency as a result of APCAs or anti-IF antibodies were excluded, since their vB_{12} deficiency cannot be directly and solely attributed to *H. pylori* infection (3–6; see Section 2.2).

In this work, we investigated the correlation between serum anti-*H. pylori* IgA antibodies and vB_{12}. The role of contaminating variables on this correlation was explored as well.

2. Methods and materials

2.1. Patients

Blood samples were collected in 5-mL tubes from 133 vB_{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_{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, antiacid, 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 µL 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 µL of conjugate for 30 min. Afterwards, substrate (100 µL) 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 APCA 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 vB12 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 Immundiagnostica; 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 µL) were mixed with 0.99 mL IgA diluents. Then 100 µL 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 µL diluted washing buffer. Except for blank wells, 100 µL 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 µL) 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 µL) 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

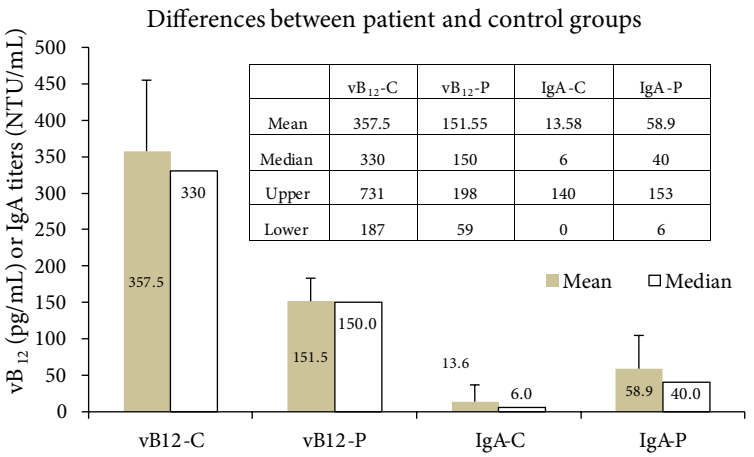
Levels of vB_{12} and anti- *Helicobacter pylori* IgA in sera are presented in Figure 1 and the Table. Means and medians for each of the four groups of subjects were obtained. The median of serum vB_{12} level for patients (150; range 59–198 pg/mL serum) was significantly different from the median for 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-*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-H. pylori IgA

Pearson's correlation coefficient 'r' between serum vB_{12} and anti-*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



	vB_{12}-C	vB_{12}-P	IgA-C	IgA-P
Mean	357.5	151.55	13.58	58.9
Median	330	150	6	40
Upper	731	198	140	153
Lower	187	59	0	6

Figure 1. Vitamin B_{12} (vB_{12}; pg/mL) and anti-*H. pylori* (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.

Table. Summary table of data statistics: Pearson's correlation (r) for raw samples.

Data exclusion	Subject group	Sample size (n)	Median	Lower–upper limits	Mean	SD/SEM	Pearson's correlation (r)
• Raw data	B12-C	105	330¶	187–731	357.5	97.5	−0.45*
	IgA-C	105	6Φ	0–140	13.6	23.1	
	vB_{12}-P	133	150¶	59–198	151.5	31.6	
	IgA-P	133	40Φ	6–153	59	45.7	
• Removed 15C or 13P	vB_{12}-C	105/90	335	212–665	362	97.5/92.6	−0.538* Or −0.521*
	IgA-C	105/90	5	0–14	6	23.1/3.9	
	vB_{12}-P	133/120	150	59–198	152/152	31.6/31.1	
	IgA-P	133/120	40	0–153	59/63	45.7/45	
• Removed 15C and 13P (28 subjects)	vB_{12}-C	105/90	335	212–665	362	97.5/92.6	−0.58*
	IgA-C	105/90	5	0–14	6	23.1/3.9	
	vB_{12}-P	133/120	150	59–198	152/152	31.6/31.1	
	IgA-P	133/120	40	0–153	59/63	45.7/45	
• Removed 15C + 13P + 7C	vB_{12}-C	83	326.5	212–665	379	62.8	−0.61*
	IgA-C	83	5	0–14	6	3.9	
	vB_{12}-P	120	155	59–198	161	45.1	
	IgA-P	120	40	0–153	58	32.2	

*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 vB_{12} values (537–665 pg/mL).
Φ,¶ Mann–Whitney differences at $P < 0.05$ and $P < 0.01$; $P = 0.00001$.

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 vB_{12} 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. vB_{12} stores

The liver is the main vB_{12} store; it stores 80% of total body vB_{12} (2 to 5 mg). Stored vB_{12} will last an adult individual for 3–5 years in the absence of significant vB_{12} intake, or for 5–6 years when vB_{12} intake is insufficient. In addition, vB_{12} 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 vB_{12} 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 vB_{12} deficiency, indicating that *H. pylori* infection preceded vB_{12} deficiency and suggesting that *H. pylori* infection may indeed have contributed to vB_{12} deficiency or caused it. The results suggest the existence of another mechanism for the role of *H. pylori* in vB_{12} deficiency other than APCAs or anti-IF antibodies. Although accumulating evidence suggests that *H. pylori* infection has a negative effect on the absorption of vB_{12} (12), the inhibition of vB_{12} 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

between vB_{12} deficiency and *H. pylori.* Subjects with anti-IF activity or APCAs were therefore excluded, since vB_{12} in these subjects cannot be directly correlated to *H. pylori* infection and would have acted as contaminating variables. The study was further strengthened by excluding patients consuming supplementary vB_{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_{12} deficiency to *H. pylori* infection. Kaptan et al. (15) found *H. pylori* in 77 (56%) of vB_{12}-deficient patients; others predicted correlation or a cause–effect relationship between vB_{12} deficiency and *H. pylori* infection, with possible destruction of parietal cells as a result of *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 *H. pylori* infections as indicated by serum levels of anti-*H. pylori* IgA and vB_{12} deficiency was affected by contaminating variables; a fraction of the control group (15/105; 14.3%) showed high IgA titers, suggesting possible asymptomatic *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_{12} (536–731 pg/mL serum; Figure 2A) increased the correlation coefficient to r = –0.61. All correlations between vB_{12} and IgA were significant; P = 0.00001.

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_{12} and anti-*H. pylori* IgA. Cluster A reflects normal healthy subjects with high vB_{12} (>200 pg/mL) and low IgA (<15 NTU/mL). Cluster B contains healthy controls with high vB_{12} but with elevated anti-*H. pylori* IgA (>15 NTU/mL), which may be indicative of asymptomatic infection or recent exposure. Among patients with mild vB_{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 *H. pylori* variants may render the anti-*H. pylori* IgA determination kit insensitive to some antigenic variants of *H. pylori* (18,20–23), i.e. IgA kit cannot equally detect all *H. pylori* antigenic variants. Second, these patients, or some of them, cannot mount

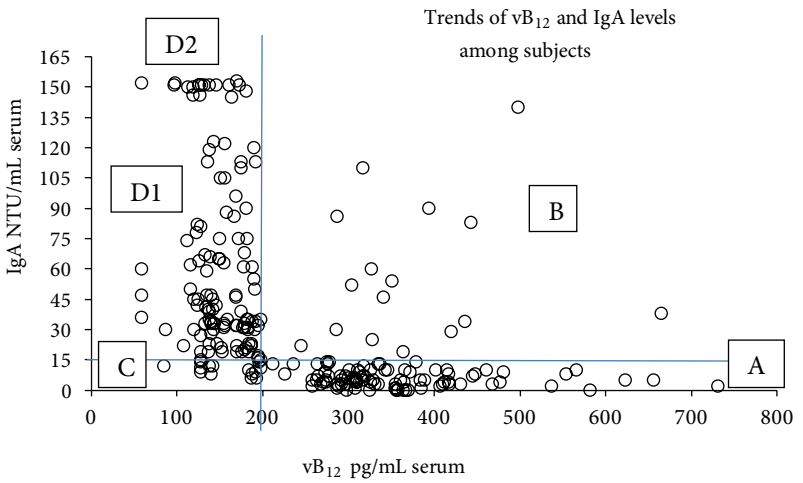


Figure 2. Scatter blot of all 238 subjects; vB_{12} was blotted against its cognate IgA value. The cutoff value for IgA (15 NTU/mL) (y = 15) and the cutoff value for vB12 (x = 200 pg/mL) are indicated. Accordingly, several clusters/trends (A–D) can be distinguished. **A**: High vB_{12} and low IgA (typical normal healthy control subjects), **B:** high vB_{12} and high IgA (possible asymptomatic patients; a potential contaminating factor, see Section 4), **C:** low vB_{12} and low IgA (patients not responding to infection; another potential contaminating factor), **D**: low vB_{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).

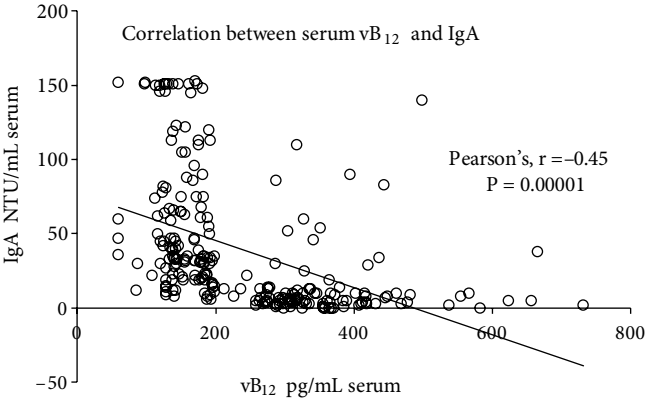


Figure 3. Correlation between vB_{12} levels and H. pylori infection (IgA). Scatter distribution of vB_{12} vs. IgA for all 238 subjects. A negative Pearson's correlation was established (r = –0.45 for original data; P < 0.05 and P < 0.01; P = 0.00001).

a significant IgA immune response against *H. pylori* (i.e. they are immune-tolerant). Third, vB_{12} 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 vB_{12} 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 vB_{12} deficiency. It is our recommendation that subjects showing high IgA titers should be treated for *H. pylori*, monitored, and prophylactically treated for vB_{12} 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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