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Simultaneous and alternative IgG seroreactivity against *Helicobacter pylori* antigens VacA, 30 kDa and 50 kDa is a better biomarker approach for the outcome of infection than VacA and 50 kDa alone

Istovremena i alternativna IgG seroreaktivnost protiv *Helicobacter pylori* antigena VacA, 50 kDa i 30 kDa je bolji biomarkerski model ishoda infekcije nego VacA i 50 kDa pojedinačno

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Abstract

Background/Aim. In our previous study, IgG seropositivities against *Helicobacter* (*H*) *pylori* antigens VacA, 50 kDa, 30 kDa, and 26 kDa were highlighted as biomarkers for the specific outcome of infection. We designed and conducted this study in order to investigate whether synchronous and/or alternative seroreactivity against *H. pylori* antigens VacA, 50 kDa, 30 kDa and 26 kDa in patients with gastric cancer and peptic ulcers exhibit stronger association than with dyspepsia and *vice versa*. **Methods.** In order to determine IgG antibodies to *H. pylori* antigens, a Western blot test was performed in 123 patients: 31 with gastric cancer, 31 with duodenal ulcer, 31 with gastric ulcer and 30 with functional dyspepsia. We analyzed IgG seroreactivity against four *H. pylori* antigens (VacA, 50 kDa, 30 kDa, 26 kDa) in their synchronous/alternative combination as well as seroreactivity to synchronous and alternative combinations of *H. pylori* antigens between a group with functional dyspepsia and others. The analysis of diagnostic characteristics of the best synchronous and alternative seroreactivity combination was done, and tested *versus* VacA as biomarker for gastric cancer and peptic ulcer, and 50 kDa as a biomarker for dyspepsia. **Results.** VacA seropositivity or 50 kDa seronegativity ($p = 0.015$) and VacA seropositivity or 50 kDa and 30 kDa seronegativity ($p = 0.044$) had the better diagnostic characteristics with statistically significantly better fraction correct than VacA seropositivity alone. VacA seronegativity along with50 kDa and 30 kDa seropositivity ($p = 0.003$), 50 kDa seropositivity ($p = 0.01$), 30 kDa seropositivity ($p = 0.015$) and 50 kDa or 30 kDa seropositivity ($p = 0.02$) had better diagnostic characteristics and significantly better fraction correct than 50 kDa seropositivity alone. **Conclusion.** Simultaneous and alternative IgG seroreactivity/unreactivity against *H. pylori* antigens VacA, 50 kDa and 30 kDa have stronger association with the specific infection outcome, considering gastric cancer and peptic ulcer, or dyspepsia, than VacA and 50 kDa IgG seropositivity alone.

Key words:
antigens; biomarkers; duodental ulcer; helicobacter pylori; stomach neoplasms; stomach ulcer.

Apstrakt

Uvod/Cilj. U našoj prethodnoj studiji IgG seropozitivnosti prema *Helicobacter* (*H*) *pylori* antigenima VacA, 50 kDa, 30 kDa i 26 kDa označene su kao biomarkeri specifičnog ishoda infekcije. Cilj ovog rada bio je da se istraži da li istovremena i/ili alternativna seroreaktivnost protiv *H. pylori* antigena VacA, 50 kDa, 30 kDa i 26 kDa ima jaču povezanost sa karcinomom želuca i peptičkim ulkusima nego sa dispepsijom, i suprotno. **Metode.** U cilju određivanja IgG antitela specifičnih prema *H. pylori* antigenima primenjen je *Western blot* test kod 123 ispitanika: 31 sa karcinomom želuca, 31 sa ulkusom duodenuma, 31 sa ulkusom želuca i 30 sa funkcionalnom dispepsijom. Analizirana je seroreaktivnost protiv četiri *H. pylori* antigena (VacA, 50 kDa, 30 kDa, 26 kDa) u njihovim istovremenim/alternativnim kombinacijama kao i istovremena i alternativna seroreaktivnost protiv *H. pylori* antigena u grupi ispitanika sa dispepsijom, u odnosu na druge grupe ispitanika. Uradjena je analiza dijagnostičkih karakteristika najboljih kombinacija istovremenih i alternativnih seroreaktivnosti i testiranje u odnosu na VacA kao biomarker karcinoma želuca i peptičkog ulkusa i 50 kDa kao biomarker dispepsije. **Rezultati.** Seropozitivnost prema VacA ili seronegativnost prema 50 kDa ($p = 0,015$) i seropozitivnost prema VacA i seronega

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tivnost prema 50 kDa ili 30 kDa ($p = 0,044$) imali su bolje dijagnostičke karakteristike sa statistički značajno boljom tačnom frakcijom u odnosu na seropozitivnost na sam VacA. Seronegativnost prema VacA zajedno sa 50 kDa i 30 kDa seropozitivnošću ($p = 0,003$), 50 kDa seropozitivnošću ($p = 0,01$), 30 kDa seropozitivnošću ($p = 0,015$), 50 kDa ili 30 kDa seropozitivnošću ($p = 0,02$) imale su bolje dijagnostičke karakteristike i statistički značajno bolju tačnu frakciju u odnosu na samu 50 kDa seropozitivnost. **Zaključak.** Istovremena i alternativna IgG seroreaktivnost/nereaktivnost protiv *H. pylori* antigena VacA, 50 kDa i 30 kDa ima jaču povezanost sa specifičnim ishodom infekcije kod karcinoma želuca i peptičkih ulkusa ili dispepsije, u odnosu na pojedinačnu IgG seropozitivnost prema VacA i 50 kDa.

Ključne reči:
antigeni; biološki pokazatelji; duodenum, ulkus; helicobacter pylori; želudac, neoplazme; želudac, ulkus.

Introduction

Because of the differences in bacterial epitopes and host characteristics, infections with *Helicobacter* (*H*) *pylori* induce different immune responses [1]. Immune response against *H. pylori* shows variability in gastric diseases in the sense of different seroreactivity to some of *H. pylori* antigens. *H. pylori* isolates from gastric tumoral mucosa and nontumoral gastric mucosa do not display the same antigens [2]. In our previous article, we reported IgG seroreactivity against *H. pylori* VacA as a potential biomarker for gastric cancer (GCA), gastric ulcer (GU) and duodenal ulcer (DU). On the other hand, IgG seroreactivity against *H. pylori* 50 kDa antigen could be a biomarker for functional dyspepsia (FD), IgG seroreactivity against *H. pylori* 30 kDa antigen could be a biomarker for antrum predominant gastritis while IgG seroreactivity against *H. pylori* Urease A 26 kDa antigen could be a biomarker for pangastritis [3]. Synchronous seropositivity and seronegativity to two or three *H. pylori* antigens were associated with the infection outcome.

The synchronous presence of IgG against 19 kDa and 35 kDa [4], 19.5 kDa and 136 kDa [5], 19.5 kDa along with 33 kDa and 136 kDa [5], 116 kDa and 89 kDa [6], the abscence of 19 kDa and 35 kDa seroreactivity [7], the presence of 120 kDa and 85 kDa [8], all were associated with GCA. The synchronous seropositivity to 87 kDa and 35 kDa [9], 125 kDa along with 87 kDa and 35 kDa [9], CagA and 35 kDa [10] all were associated with peptic ulcer (PU). On the other hand, synchronous seropositivity to 120 kDa, 33 kDa, 30 kDa and 19 kDa was associated with the intensity of antral inflammation [11].

We conducted this study with the aim to investigate the association of synchronous and alternative seroreactivity to VacA, 50 kDa, 30 kDa and 26 kDa. VacA and 26 kDa seropositivity were considered as a biomarker for the serious outcome of infection in patients with GCA and PU, and, on the other hand, seronegativity was considered as a biomarker for FD. 50 kDa seropositivity was considered as a biomarker for FD and 30 kDa, and, as opposed to that, seronegativity was considered as a biomarker for the serious outcome of infection. At the same time, it was interesting to compare the degree of association of VacA seropositivity and 50 kDa seropositivity with the specific outcome of infection vs. synchronous and alternative seroreactivity to two or three *H. pylori* antigens.

Methods

The study was conducted during 2009 at the Clinic for Gastroenterology and Hepatology of the Military Medical Academy (MMA), Institute of Pathology of the MMA and Institute of Microbiology of the MMA, Belgrade, Serbia. We selected and enrolled the patients with dyspeptic symptoms, different underlying disease (GCA, DU, GU and gastritis), and actual *H. pylori* infection confirmed by histopathological examination and anti-*H. pylori* IgG positive ViraBlot.

We took medical history from all patients and performed a physical examination, abdominal ultrasound (US) or computer tomography (CT), esophagogastroduodenoscopy (EGDS), complete blood count (CBC), liver and renal chemistry. The inclusion criteria were: 1) the presence of dyspepsia symptoms, 2) previously untreated patients due to *H. pylori* infection, 3) without treatment with proton pump inhibitors and H2 blockers in the last two weeks, 4) the absence of malignancy except for GCA, 5) the absence of any immunological disorder, 6) informed consent of the patient for: a) EGDS and biopsy, b) blood sample for analyses, c) participation in the study, 7) endoscopic and histopathological diagnosis of one of the following diseases: GCA, DU, GU, gastritis; 8) confirmed histopathological diagnosis of *H. pylori* infection, 9) Western blot detection system (ViraBlot) IgG positive for *H. pylori* infection.

EGDS was performed in all our patients in the endoscopy section using Olympus (GIFQ165, SN: 2207997, Olympus corporation, Tokyo) forward viewing EGDS under local application of Xylocaine spray. A minimum of four gastric mucosal tissue biopsies (two of them from the antrum and corpus) and additional biopsies from any endoscopically visible lesion were taken. All patients were examined for findings that indicated endoscopic gastritis, such as erythema, hyperemia, atrophy, and mucosal nodularity according to the criteria of the Houston-updated Sydney grading system, and for gastric tumor, DU and GU.

Blood samples were obtained from all of them and frozen at -20 °C degree. Using the ViraBlot, IgG anti Vacuolating cytotoxin A (VacA) 87 kDa, Cytotoxin associated with gene A (CagA) 136 kDa, Urease B 66 kDa (UreB 66), Heat shock protein 60 kDa (Hsp60), Flagellin 55 kDa (Fla 55), 50 kDa, 30 kDa, Urease A 26 kDa (UreA 26) and 24 kDa *H. pylori* antigens were identified. *H. pylori* antigens of ViraBlot represent a combination of German patient isolates of highly antigenic *H. pylori* strains. Bands for diagnosis of *H. pylori* infection were divided into highly specific as CagA 136 kDa, VacA 87 kDa, 30 kDa, UreA 26 kDa, 24 kDa and less specific as Hsp 60 kDa and 50 kDa. According to the manufacturer's guideline for use, the test was considered negative if there were no bands or there were nonspecific bands such as

UreB 66 kDa, Hsp 66 kDa, Fla 55 kDa, 50 kDa. The test was possibly positive if there was one clear specific band of 30 kDa, UreaA 26 kDa, 24 kDa. The test was positive if there was at least one band of following two specific CagA 136 kDa or VacA 87 kDa or at least one clear band of 30 kDa, UreaA 26 kDa, 24 kDa or one clear band of 30 kDa, UreA 26 kDa, 24 kDa and one clear band of Hsp60 kDa, and 50 kDa. In this study we used the data from our previous study [3], considering only ViraBlot IgG against VacA, 50 kDa, 30 kDa and 26 kDa.

The diagnosis of GCA was established in 31 patients, DU in 31 patients, and GU in 31 patients, while in 30 patients gastritis with FD was diagnosed.

All patients included in the study were analyzed in several ways. Firstly, the patients were analyzed according to the baseline diagnosis: GCA, GU, DU and dyspepsia. Subsequently, groups with GCA and PU were joined in one group and tested *vs.* the group of dyspepsia.

In the first analysis, we searched for combination seropositivity as a biomarker for the serious outcome of infection in baseline groups in the following combinations: VacA seropositivity along with the absence of 50 kDa seropositivity; VacA seropositivity along with the absence of 30 kDa seropositivity; VacA seropositivity along with 26 kDa seropositivity; the abscence of both 50 and 30 kDa seropositivity; the abscence of 50 kDa and presence of 26 kDa seropositivity; the absence 30 kDa and presence of 26 kDa seropositivity.

In the second part of the first analysis, we searched for combination seropositivity as a biomarker for FD: the absence of VacA seropositivity and the presence of 50 kDa seropositivity; the absence of VacA and the presence of 30 kDa seropositivity; the absence of VacA and the presence of 50 and 30 kDa seropositivity; the presence of 50 kDa and 30 kDa seropositivity together.

In the second analysis, we searched for alternative combination of seropositivity/negativity as biomarkers for the serious outcome in baseline groups in the following combinations: VacA seropositivity or 50 kDa seronegativity; VacA seropositivity or 50 kDa and 30 kDa seronegativity; VacA seropositivity along with 26 kDa seropositivity and 50 kDa seronegativity; VacA seropositivity, 26 kDa seropositivity, 30 kDa seronegativity.

In the second part of the second analysis, we searched for the alternative combination of seropositivity/negativity as a biomarker for FD in the following combinations: VacA seronegativity along with 50 kDa or 30 kDa seronegativity; 50 kDa or 30 kDa seropositivity.

In the third analysis, we joined groups with GCA, GU and DU and tested *vs.* FD with the same criteria as in the first and second analysis.

We performed statistical analysis of the frequency of the previously mentioned combinations in baseline groups GCA, GU, DU *vs.* FD and GCA and PU group *vs.* FD group.

In the fourth analysis, accuracy and discriminative ability of various seropositive/seronegative combination against investigated *H. pylori* antigens in the prediction of the outcome of infection were estimated with Sensitivity (Se), Specificity (Sp), Positive predictive value (PPV), Negative predictive value (NPV), Fraction correct (FC) and Clinical utility index (CUI) in the form of Case-finding utility or Positive utility index (CUI Ve+) and Screening utility or Negative utility index (CUI Ve-). CUI Ve+ = Se x PPV and CUI Ve- = Sp x NPV represent important indexes for clinicians estimating both accuracy and discriminative ability of the test.

Statistical analysis

Complete statistical data analysis was done with the statistical software package, SPSS Statistics 18. Most of the variables were presented as the frequency of certain categories, so *t*-test of proportion or cross-tabulation analysis (odds ratio, confidence intervals) were done for the calculation of statistical significance of the differences between groups. In case of continuous data, variables were presented as median, minimal and maximal values (range). All the analyses were estimated at minimal $p < 0.05$ level of statistical significance.

Results

Four groups of patients with GCA, DU, GU and upper FD were comparable regarding gender and age (Table 1).

Seroreactivity against synchronous combinations of *H. pylori* antigens VacA, 50 kDa, 30 kDa and 26 kDa is shown in Table 2.

Seropositivity to VacA and seronegativity to 50 kDa taken together were significantly more frequent in GCA ($p = 0.045$), GU ($p = 0.02$) and DU ($p = 0.045$) than in FD.

Seronegativity to both 30 kDa and 50 kDa was significantly more frequent in GCA ($p = 0.006$), GU ($p = 0.006$) than in FD, but with only trend to significance in comparison with DU ($p = 0.056$).

Table 1

Demographic and clinical characteristics of the examined patients

Parameters	Groups of patients				Total (n = 123)
	GCA (n = 31)	DU (n = 31)	GU (n = 31)	FD (n = 30)	
Gender, n					
male	10	13	12	13	48
female	21	18	19	17	75
Age (years), median (range)	65 (40–85)	54 (21–87)	67.5 (34–81)	63.5 (21–80)	63 (21–87)

GCA – gastric cancer; DU – duodenal ulcer; GU – gastric ulcer; FD – upper functional dyspepsia.

Table 2

Seroreactivity against synchronous combinations of *H. pylori* antigens VacA, 50 kDa, 30 kDa and 26 kDa in four groups of patients

WB IgG	Groups, n (%)					FD vs. others		
	GCA 31 (100)	DU 31 (100)	GU 31 (100)	FD 30 (100)		GCA	DU	GU
VacA+ 50 kDa-	8 (26)	8 (26)	9 (29)	2 (7)	*p*	0.045	0.045	0.02
					OR			
					CI			
VacA+ 30 kDa-	5 (16)	4 (13)	7 (23)	3 (10)	*p*	ns	ns	ns
					OR			
					CI			
VacA+ UreA26+	15 (48)	17 (55)	15 (48)	10 (30)	*p*	ns	ns	ns
					OR			
					CI			
VacA- 50 kDa+	6 (19)	5 (16)	6 (19)	15 (50)	*p*	0.01	0.005	0.01
					OR	4.2	5.2	4.2
					CI	1.3–13.1	1.6–17.2	1.3–13.1
VacA- 30 kDa+	5 (16)	10 (32)	4 (13)	16 (53)	*p*	0.002	ns	0.0008
					OR	5.9		7.7
					CI	1.8–19.6		2.2–27.5
VacA- 50 kDa+ 30 kDa+	2 (6)	5 (16)	3 (10)	11 (37)	*p*	0.005	ns	0.02
					OR			
					CI			
50 kDa+ 30 kDa+	12 (39)	12 (39)	7 (23)	17 (57)	*p*	ns	ns	0.006
					OR			0.22
					CI			0.09–0.68
50 kDa– 30 kDa-	10 (32)	6 (19)	10 (32)	1 (3)	*p*	0.006	ns	0.006
					OR			
					CI			
50 kDa- 26 kDa+	9 (29)	14 (45)	14 (45)	4 (13)	*p*	ns	0.01	0.01
					OR			
					CI			
30 kDa- 26 kDa+	11 (35)	5 (16)	10 (32)	6 (20)	*p*	ns	ns	ns
					OR			
					CI			

WB – Western blot; OR – odds ratio; CI – confidence intervals; ns – non significant.
For other abbreviations see under Table 1.

Seronegativity to 50 kDa along with 26.5 kDa seropositivity was significantly more frequent in DU ($p = 0.01$) and GU ($p = 0.01$) than in FD, but not in GCA.

Seronegativity to VacA along with 50 kDa seropositivity was significantly more frequent in FD than in GCA ($p = 0.01$), GU ($p = 0.01$), and DU ($p = 0.005$).

Seronegativity to VacA along with 30 kDa seropositivity was significantly more frequent in FD than in GCA ($p = 0.002$) and GU ($p = 0.0008$), but not than in DU.

Seronegativity to VacA along with 50 kDa and 30 kDa seropositivity was significantly more frequent in FD than in GCA (0.005) and GU ($p = 0.02$), but showed only trend to significance in comparison with DU ($p = 0.068$).

Seropositivity to both 50 kDa and 30 kDa was significantly more frequent in FD than in GU ($p = 0.006$), but not as frequent as in GCA and DU.

The other synchronous seropositivity combinations were not different among the investigated groups (Table 2).

All alternative seropositivity/seronegativity combinations, apart from 50 kDa or 30 kDa seropositivity, which is significantly more frequent in FD than in GCA ($p = 0.006$) and GU ($p = 0.006$), but not as frequent as in DU, were significantly more frequent in GCA, PU, GU groups than in FD or *vice versa* depending on the intention of the test (Table 3).

Synchronous seropositivity to VacA with seronegativity to 50 kDa ($p = 0.014$), synchronous seronegativity to both 50 kDa and 30 kDa ($p = 0.0024$), and 26 kDa seropositivity with seronegativity to 50 kDa ($p = 0.013$) were significantly more frequent in GCA and PU than in FD. Synchronous combinations of VacA seronegativity with 50 kDa seropositivity ($p = 0.001$), 30 kDa seropositivity ($p = 0.01$), 50 kDa and 30 kDa seropositivity ($p = 0.01$) were significantly more frequent in FD than in GCA and PU. In addition to that seropositivity combination of 50 kDa and 30 kDa was significantly more frequent in FD ($p = 0.039$) (Table 4).

All alternative combinations, except for VacA seropositivity or 26 kDa seropositivity, had a significantly more different frequency than the comparison group. Other VacA seropositive combination whatever companion (50 kDa seronegativity, 30 kDa seronegativity, both 50 kDa and 30 kDa seronegativity, 26 kDa seropositivity with either 50 kDa or 30 kDa seronegativity) were significantly more frequent in GCA than in FD. VacA seronegativity along with 50 kDa or 30 kDa seropositivity ($p = 0.0001$), and 50 kDa or 30 kDa

Table 3

Seroreactivity against alternative combination of *Helicobacter pylori* antigens VacA, 50 kDa, 30 kDa and 26 kDa in four groups of patients

WB IgG	Groups, n (%)					FD *vs* others		
	GCA 31 (100)	DU 31 (100)	GU 31 (100)	FD 30 (100)		GCA	DU	GU
VacA + or 50kDa-	24 (77)	26 (84)	25 (81)	14 (47)	*p* OR CI	0.013 3.92 1.3–11.3	0.002 5.91 1.8–19.6	0.006 4.76 1.51–14.95
VacA+ or 50kDa- 30kDa-	24 (77)	20 (65)	24 (77)	10 (32)	*p* OR CI	0.0005 6.86 2.2–21.3	0.015 3.63 1.6–10.47	0.0005 6.86 2.2–21.3
VacA+ or 26kDa+ 50kDa-	21 (68)	24 (77)	23 (74)	12 (40)	*p* OR CI	0.03 3.15 1.1–9	0.003 5.14 1.7–15.7	0.007 4.31 1.45–12.8
VacA+ or 26kDa+ 30kDa-	23 (74)	20 (65)	23 (74)	11 (37)	*p* OR CI	0.003 4.96 1.67–14.84	0.03 3.14 1.1–8.93	0.003 4.96 1.67–14.84
VacA- 50kDa+ or 30kDa+	8 (26)	10 (32)	6 (19)	18 (60)	*p* OR CI	0.007 4.3 1.5–12.8	0.03 3.2 1.1–9	0.001 6.3 2–19.8
50kDa+ or 30kDa+	21 (68)	26 (84)	21 (68)	29 (97)	*p* OR CI	0.006	ns	0.006

WB – Western blot; OR – odds ratio; CI – confidence intervals; ns – non significant.
For other abbreviations see under Table 1.

Table 4

Seroreactivity against synchronous combination of *Helicobacter pylori* antigens in patients with gastric cancer (GCA) and peptic ulcers (PU), and functional dyspepsia (FD)

WB IgG	Patients, n (%)		*p*	OR	CI (95%)
	GCA and PU 93 (100)	FD 30 (100)			
CagA + VacA+	50 (54)	9 (30)	0.023	2.71	1.1–6.5
VacA+ 26kDa+	47 (50)	10 (30)	ns		
VacA+ 50kDa-	25 (27)	2 (7)	0.03	5.1	1.1–23
VacA+ 30kDa-	16 (17)	3 (10)	ns		
VacA- 50kDa +	17 (18)	15 (50)	0.0006	4.5	1..8–10.9
VacA- 30kDa+	19 (20)	16 (53)	0.0006	4.5	1.9–10.7
VacA- 50kDa+ 30kDa+	10 (11)	11 (37)	0.001	4.8	1.8–12.9
50kDa+ 30kDa+	31 (33)	17 (57)	0.03	2.6	1.1–6.1
50kDa- 30kDa-	26 (28)	1 (3)	0.004	11.2	1.5–87
26kDa+ 50kDa-	37 (40)	4 (13)	0.01	4.5	1.4–13.3

WB – Western blot; OR – odds ratio; CI – confidence intervals; ns – non significant.

Table 5

Seroreactivity against alternative combination of *Helicobacter pylori* antigens in patients with gastric cancer (GCA) and peptic ulcers (PU), and functional dyspepsia (FD)

WB IgG	Patients, n (%)		*p*	OR	CI (95%)
	GCA and PU 93 (100)	FD 30 (100)			
VacA+ or 50kDa-	74 (80)	14 (47)	0.0005	4.45	1.85–10.7
VacA+ or 26kDa+	82 (88)	27 (90)	ns		
VacA+ or (50kDa-)+(30kDa-)	68 (73)	10 (32)	0.0002	5.44	2.24–13.2
VacA+ or (26kDa+)+(50kDa-)	68 (73)	12 (40)	0.002	4.08	1.7–9.67
VacA+ or (26kDa+)+(30kDa-)	66 (71)	11 (37)	0.0016	4.22	1.77–10.05
(VacA-)+(50kDa+) or (30KDa+)	24 (26)	20 (67)	0.0001	5.8	2.4–14
30kDa+ or 50kDa+	68 (73)	29 (97)	0.031	10.7	1.4–82

WB – Western blot; OR – odds ratio; CI – confidence intervals; ns – non significant.
For other abbreviations see under Table 1.

Table 6

Diagnostic characteristics of IgG against 50 kDa, synchronous and alternative combination of VacA, 50 kDa, 30 kDa for functional dyspepsia (FD)

WB bands antigen seroreactivity	GCA and PU, n (%), (n = 93)	FD, n (%) (n = 30)	Sn	Sp	PPV	NPV	CUI+	CUI-	Fraction correct
50 kDa	44 (47)	23 (77)	77	53	34	87	0.26 (VP)	0.46 (P)	61
VacA- 50 kDa+ 30 kDa+	10 (11)	11 (37)	37	89	52	81	0.19 (VP)	0.73 (G)	76**
VacA- 50 kDa+	17 (18)	15 (50)	50	82	47	83	0.23 (VP)	0.68 (G)	74**
VacA- 30 kDa+	19 (20)	16 (53)	53	80	46	84	0.24 (VP)	0.67 (G)	73*
VacA- 50 kDa+ or 30 kDa+	24 (26)	20 (67)	67	74	45	87	0.30 (VP)	0.65 (G)	72.4*

***p < 0.05; **p < 0.01 vs. 50 kDa group.**
Sn – sensitivity; Sp – specificity; PPV – positive predictive value; NPV – negative predictive value; CUI – clinical utility index.
For other abbreviations see under Table 1.
A qualitative interpretation of the clinical utility index: (E) => 0.81 excellent; (G) => 0.64 good; (SA) => 0.49 satisfactory/adequate; (P) => 0.36 poor; (VP) < 0.36 very poor.

seropositivity (p = 0.031) were significantly more frequent in FD than in GCA and PU (Table 5).

Among synchronous and alternative seropositivity/seronegativity combinations, we selected those with the most pronounced probability and/or odds ratio and compared their diagnostic characteristics with 50 kDa or VacA. VacA seropositivity or 50 kDa seronegativity (p = 0.015) and VacA seropositivity or 50 kDa and 30 kDa seronegativity (p = 0.044) had better diagnostic characteristics with statistically significant better fraction correct than VacA seropositivity alone (Table 6).

VacA seronegativity along with either 50 kDa or 30 kDa seropositivity (p = 0.003), 50 kDa seropositivity (p = 0.01), 30 kDa seropositivity (p = 0.015), 50 kDa or 30 kDa seropositivity (p = 0.02) had better diagnostic characteristics and significantly better fraction correct than 50 kDa seropositivity alone (Table 7, Figure 1).

Table 7

Diagnostic characteristics of IgG against VacA, synchronous and alternative combination of VacA, 50 kDa, 30 kDa for GCA and PU

WB bands antigen seroreactivity	GCA and PU, n (%), (n = 93)	FD, n (%), (n = 30)	Sn	Sp	PPV	NPV	CUI+	CUI-	Fraction correct
VacA	53 (57)	10 (33)	57	67	84	33	0.48 (P)	0.22 (VP)	59
VacA+ or 50 kDa-	75 (82)	14 (47)	81	53	84	47	0.68 (G)	0.25 (VP)	74*
VacA+ or 50 kDa- 30 kDa-	68 (73)	10 (33)	73	67	87	44	0.64 (G)	0.3 (VP)	71.5*
VacA+ or 26 kDa+ 50 kDa-	68 (73)	12 (40)	73	60	85	42	0.62 (SA)	0.25 (VP)	69.9
VacA + or 26 kDa+ 30 kDa -	66 (71)	11 (37)	71	63	86	41	0.61 (SA)	0.26 (VP)	69

***p* < 0.05 vs. VacA group.**

Sn – sensitivity; Sp – specificity; PPV – positive predictive value; NPV – negative predictive value; CUI – clinical utility index. For other abbreviations see under Table 1.

A qualitative interpretation of the clinical utility index: (E) => 0.81 excellent; (G) => 0.64 good; (SA) => 0.49 satisfactory/adequate; (P) => 0.36 poor; (VP) < 0.36 very poor.

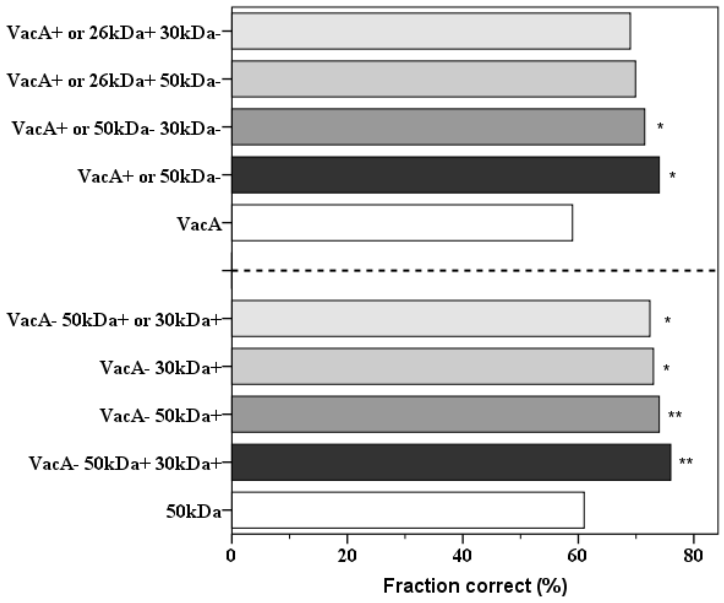

Fig. 1 – Fraction correct of different seroreactivity combination against *Helicobacter pylori* VacA, 50 kDa, 30 kDa and 26 kDa in comparison with 50 kDa and VacA alone.

***p* < 0.05; ****p* < 0.01 vs. corresponding 50 kDa (VacA) group.**

Discussion

Difference in immune response with preferences to specific antigens was observed in young children [12], but in GCA and PU also, with different results [3–11]. Our analysis of synchronous seropositivity/seronegativity IgG combinations as a candidate biomarker for the serious outcome of the infection showed that VacA+50 kDa- is significantly more frequent in patients with GCA and PU than in FD due to very low frequency in FD (7%).

At the same time, the frequency of this combination in GCA and PU group was also relatively low (27%), and its application in clinical practice seems implausible. Seronegativity to both 50 kDa and 30 kDa had similar results.

Alternative seropositivity/seronegativity combination could offer the manifestation of the existing differences. For example, VacA seronegativity was present in 25% of patients with either GCA or PU with synchronous 50 kDa seronegativity. If we applied criteria for VacA seropositivity or 50 kDa seronegativity, we would increase the percent of true positive finding for 25%, and, at the same time, the specificity would decrease due to 13.3% false positive results in FD. The analysis of an alternative seropositivity/seronegativity candidate biomarker for the serious outcome showed more

interesting results with 4 promising combination of VacA+ or 50 kDa- (p = 0.0005; OR = 4.45); 50 kDa+ and 30 kDa- (p = 0.0002; OR = 5.54); 26 kDa+ and 50 kDa-, and 26 kDa+ and 30 kDa-. These results were notably interesting, giving us the idea to test their diagnostic abilities. When we analyzed diagnostic characteristics of the above-mentioned combinations, two of them VacA+ or 50 kDa- (p = 0.015) and VacA+ or 50 kDa+ and 30 kDa- (p = 0.044) showed significantly better results than VacA alone. VacA+ or 50 kDa- had the highest sensitivity (81%), lower specificity than VacA+ or 50 kDa- and 30 kDa- (53% vs. 67% vs. 67%, respectively); lower PPV than VacA+ or 50 kDa- and 30 kDa- (84 vs. 87, respectively); highest NPV (47 vs. 33 vs. 44, respectively), and the highest CUI+ (0.68 vs. 0.48 vs. 0.64, respectively). CUIVe+ of both tests can be considered as good, but CUIVE- is very poor for both. These two tests may perspectively have a good role in clinical practice [13, 14]. The analysis of synchronous and alternative seropositivity/seronegativity of VacA, 50 kDa, 30 kDa, 26 kDa antigen combinations regarding a biomarker for functional dyspepsia showed four significant results. VacA seronegativity along with 50 kDa seropositivity (p = 0.006, OR = 4.5), 30 kDa seropositivity (p = 0.0006, OR = 4.5), 50 kDa and 30 kDa seropositivity (p = 0.001, OR = 4.8), and, apart from that, 50 kDa and 30 kDa seropositivity together, were more frequent in FD than in GCA and PU groups. Two alternative combinations have been analyzed, VacA- along with 50 kDa+ or 30 kDa+ (p = 0.0001, OR = 5.8), 50 kDa+ or 30 kDa+ (p = 0.031, OR = 10.7), both being significantly more frequent in FD than in GCA

The four combinations with the best results as a biomarker for FD: VacA- along with either 50 kDa+ and 30 kDa+, 50 kDa+ and 30 kDa+, 50 kDa+ or 30 kDa+ have significantly better diagnostic characteristics than 50 kDa seropositivity alone. When we compared diagnostic characteristics of these four tests with 50 kDa seropositivity, we found that sensitivity and NPV were the highest in 50 kDa. Specificity, PPV and CUIVE- were the highest in VacA- along with 50 kDa+ and 30 kDa+ combination. NPV and CUIVE+ were the highest in VacA- along with 50 kDa+ or 30 kDa+. It is interesting that all four combinations have CUIVE- at the level of "good", but at the same time all CUIVE+ were "very poor". This implies possible application of these seroreactivity combinations as a test with good screening utility. Alternative seropositivity/seronegativity combination led to the improvement of sensitivity, with no significant deterioration of specificity.,

Previously published results highlighted the association of 19 kDa or 19.5 kDa seropositivity with 35 kDa [4], 136 kDa [5], 136 kDa along with 33 kDa [5] and GCA. Contrary to the previous, Chua et al. [7] reported that the seronegativity of 19 kDa and 35 kDa were associated with GCA. According to the study of Karami et al, [6] 126 kDa and 89 kDa seropositivity are associated with GCA, but Janulaityte-Günther et al. [8] reported only the synchronous seropositivity of 120 kDa and 85 kDa associated with GCA, while with the absence of one seropositivity, the association with GCA disappeared. According to the published results, PU is associated with 35 kDa seropositivity along with either 87 kDa seropositivity, 87 kDa or 125 kDa seropositivity [9], and CagA seropositivity [10]. In our study, we did not use 19 kDa, 19.5 kDa and 35 kDa antigens. Shafaie et al. [15] reported that multiplex serology assay using synchronously more *H. pylori* antigens was able not only to detect subjects with current *H. pylori* infection, but it could also screen dyspeptic patients for the presence of gastric atrophy. This simple and cost-efficient method can supplement routine screening ELISAs to increase the chances of detecting current infections, as well as atrophic gastritis.

The limitations of our study were a relatively small number of participants, and German ViraBlot with *H. pylori* isolates from German patients.

Conclusion

In our study combined seropositivity/ seronegativity of *H. pylori* antigens VacA, 50 kDa and 30 kDa had a significantly stronger association with either GCA and PU or FD than previously reported single seropositivity against VacA and 50 kDa. It could be interesting to analyze diagnostic abilities of the observed shift in response to *H. pylori* antigens VacA, 50 kDa and 30 kDa related to the severity of gastric mucosal lesion. Synchronous and/or alternative seropositivity/seronegativity to *H. pylori* antigens VacA, 50 kDa and 30 kDa may be investigated as a diagnostic test, and in our further analysis it would be justified to be tested vs. and along with alarm features in patients with dyspeptic symptoms, in order to select the patients with the uninvestigated dyspepsia who are candidates for early EGDS.

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