Original Article

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Microbiology Section

Non-Invasive Diagnosis of *Helicobacter pylori*: Evaluation of Two Enzyme Immunoassays, Testing Serum IgG and IgA Response in the Anand District of Central Gujarat, India

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ABSTRACT

Context: Validation of an accurate and less cumbersome noninvasive method to detect current *Helicobacter pylori* infection is a requisite for any laboratory.

Objectives: The purpose of this study is to corroborate the usefulness of two commercially available kits NovaTec ELISA-A and ELISA-G, in the detection of ongoing *H.pylori* infection.

Materials and Methods: Two hundred and twenty eight consecutive serum samples of symptomatic patients who attended the endoscopy unit of "Deep" surgical hospital, Anand, which were collected during the period from 27th February 2008 to 31st august 2011, were studied. The sera were processed and tested for the detection of the *H.pylori* IgG and IgA antibody by using a solid phase; capture micro well ELISA, procured from Nova Tec immunodiagnostica GmbH Germany.

Results: IgG ELISA showed 100% sensitivity and Negative predictive value (NPV), while IgA ELISA was better in terms of specificity (61.4%) and accuracy (63%) as compared to IgG ELISA. We found 7% (16/228) of IgA positive cases with IgG negative response. IgG response was more common in reflux esophagitis patients (OR 1.451, 95%CI-0.850-2.477) and then in gastritis (OR 0.962, 95%CI-0.570-1.621) and duodenitis(OR-0.806, 95%CI-0.112-5.827), while IgA positive response was more common in duodenitis patients (OR-1.383, 95%CI-0.191-9.995) and reflux esophagitis patients (OR 1.289, 95% CI-0.756-2.197) and least in duodenal ulcer patients (OR 0.670, 95%CI-0.222-2.029).

Conclusion: IgG update is reliable and accurate test and can be expedient as a screening test and thus serve as an alternative to endoscopy. For the purpose of excluding infection with *H.pylori*, the performance of IgG is moderate (low specificity) but can be improved by conjunctional IgA testing which will offer some additional diagnostic value.

Keywords: *Helicobacter pylori*, Enzyme linked immunosorbant assay (ELISA), Immunoglobulin G (IgG), Immunoglobulin A (IgA)

INTRODUCTION

Helicobacter pylori, in a very short period of time, has become one of the most important etiological agent causing gastrointestinal infections. This organism has dramatically affected gastroenterology and has been a topic of much research. It is now felt to play major roles in the reoccurrence or pathogenesis of duodenal ulcer diseases, chronic antral gastritis, gastric ulcer diseases and, possibly, non-ulcer dyspepsia and gastric cancer [1]. The prominence of *H.pylori* makes it imperative to develop a safe, noninvasive simple method of detection [1].

The most indicative and accurate method of *H.pylori* diagnosis is endoscopy followed by colorimetric assays. Most of the laboratories in Central Gujarat confer the results mostly on the basis of IgG serology and only those experiencing intolerable symptoms with indications of progressive disease are referred for endoscopy. This method of *H.pylori* diagnosis, is quite unappealing and is met by severe hesitations due to its invasive nature. A simple cost-effective diagnostic procedure which would bypass the need for endoscopy is highly required for a population like Central Gujarat in which the rate of infection is overwhelming.

Serologic testing is the commonest method of non-invasive diagnosis for *H.pylori* used in epidemiological studies, enabling us to screen patients suffering from dyspepsia before performance of endoscopies and for long term follow-ups subsequent to drug therapy [1]. When performing serologic testing, however, the physician does need to keep in mind the sensitivity, specificity, and predictive value of the test, parameters that depend on the prevalence of the disease in the population being tested. Generally the prevalence of raised IgG in the population tends to be higher in developing countries than in developed countries [2]. When prevalence of the disease is high, a sensitive test might be chosen, whereas when the prevalence of disease is low, a specific test is more appropriate [2]. Several reports have convincingly demonstrated that changes in *H.pylori* IgG levels can be used to monitor the success of antibiotic treatment. Nearly all studies have found that successful treatment is associated with a 40-50% decrease in IgG levels by 6 months post-treatment. However, only about 25% of successfully treated patients show a complete disappearance of IgG antibodies even measured 3.5 years after treatment [3].

The clinical importance of the IgA response is underlined when considered in association with earlier findings showing an association between *H.pylori* IgA and gastric cancer, with increased risk of peptic ulcer disease, atrophic gastritis and intestinal metaplasia [3]. As the *H.pylori* IgA response may develop later in life, many *H.pylori* infected young individuals may have IgG only, although this does not exclude the possibility of developing an IgA response [3]. Commercial serological assays for *H.pylori* detection demonstrate varying accuracies for different populations which may be due to the vast molecular diversity in genes and encoded proteins among

isolated *H.pylori* strains from different geographic regions used for the coating antigen preparations [4].

In Anand District, it has been observed that majority of the individuals are suffering from dyspeptic symptoms attending the gastroenterologist, it is therefore very important to rule out the presence of *H.pylori* infection. So in the present study the corroboration of both IgG and IgA antibodies in the infected serum are done in comparison to the Gold standard tests, so as to preclude on giving false treatment to the patients.

MATERIALS AND METHODS

Subjects

Two hundred twenty eight consecutive, symptomatic patients (144 males and 84 females; age 10-90 years) attending the endoscopy unit of Deep Surgical Hospital, Anand, Gujarat, were included. 115 patients with gastritis, 94 patients with reflux esophagitis, 15 with duodenal ulcer, and 4 patients with duodenitis, based on endoscopic findings were enrolled in this study. Patients taking aspirin or non-steroidal anti-inflammatory drugs (NSAIDS) in the past 4 weeks or those on proton pump inhibitors (PPI), patients with previous therapy to eradicate *H.pylori* or if the informed consent was not obtained were excluded from the study. The study was conducted in the Department of Microbiology of Shree P. M. Patel Paramedical College, Anand, (India) during the period from February 2008 to August 2011.

Ethical considerations

Approval from Human research ethics committee (HREC) of H. M. Patel center for medical care and education, Pramukh Swami Medical College, Karamsad, was taken prior to initiation of the work. Study was done according to the principles of Helsinki Declaration. Dully filled consent form was obtained from all the patients participating in the study.

Criteria for true positive result for *H.pylori*: (Gold standard tests)

Subjects were classified as having current infection with *H.pylori* if Rapid urease test (RUT) and histology were positive along with Gram's staining or if *H.pylori* were cultured from the biopsy specimen, if any single test out of RUT, Gram staining or histology was positive then the result was considered negative.

Sampling

Four antral biopsies were collected from each symptomatic patient in Brain-heart infusion (BHI) broth and were transported to the laboratory without delay.

For Gram staining- biopsy was crushed and smears were prepared on a clean slide and stained with the standard protocols [5]. For RUT, one biopsy specimen was immediately placed into 0.5 ml urea indicator broth at room temperature. Any change in color from yellow-orange to pink in the next four hours indicated the presence of *H.pylori* in the sample [6]. *H.pylori* were isolated by streaking homogenized biopsies on Brucella blood agar(Brucella Hi Veg Agar Base, procured from Hi-Media labs) augmented with 7-10% human defibrinated blood and Skirrow's selective supplement (vancomycin, 10µg/ml; polymyxin B sulfate 2.5 IU /ml; trimethoprim lactate 5 µg/ml)(campylobacter supplement-III, Hi-Media labs). These plates were incubated at 37°C in an anaerobic jar with providing gas pack kit (campylobacter gas generating kit BR 060A, Oxoid) for *H.pylori* which provides suitable microaerophilic condition. *H.pylori* isolates were identified by typical colony morphology (minute, translucent, round, convex colonies on Brucella blood agar) and characteristic gram negative spiral appearance, positive urease, oxidase and catalase test [7].

Histology slides were stained with Giemsa and Warthin-starry and scored for the presence of *H.pylori* in several fields [8].

Serology

For serological studies 5mL of venous blood was also obtained from every subject. Serum samples were separated and stored at 4°C until ELISA was done (2 days), after which the samples were stored at 20°C. Specific IgA and IgG antibodies against *H.pylori* were measured using enzyme linked immunosorbant assay (ELISA). Kits were procured from Nova Tec immunodiagnostica GmbH, Germany.

Antigens used for IgA: recombinant Cytotoxin associated gene (CagA) - and recombinant urease-antigens. Antigens used for IgG: Highly purified proteins associated with CagA genes (120 KD) and Vacuolating (VacA) genes (87 KD) as well as urease-antigens. Procedure of ELISA was according to the manufacturer's instructions. Results were interpreted as reactive if the absorbance value is >20NTU/ml, Non-Reactive if value is <15 NTU/ml and in Grey zone if value is between 15-20 NTU/ml.

RESULTS

Of 228 patients, 18 were *H.pylori* positive and 210 were *H.pylori* negative by using the results of the biopsy test as the "Gold standard". Out of 18 positive samples EIA-G detected all the 18, while EIA-A detected 15 samples positive with 3 false negative samples. One hundred eight samples were false positive with EIA-G update while 81 were false positive with EIA-A update. This result shown in [Table/Fig-1] reveals that the EIA-G update had an optimal sensitivity and negative predictive values of 100% but with reduced specificity of 48.6%. EIA-A update performed better in terms of specificity. EIA-A yielded the most false negative results (46 samples). If we see the combine result comparing to the gold standard tests then, 80 samples were positive by both IgG and IgA while 86 samples were negative by both. Fourty-six samples were positive by IgG but negative by IgA and 16 samples were positive by IgA but negative by IgG [Table/Fig-2].

Antibody responses in various gastro duodenal diseases [Table/Fig-3]:

IgG response was more common in reflux esophagitis patients (OR 1.451, 95%CI-0.850-2.477) and then in gastritis (OR 0.962, 95%CI-0.570-1.621) and duodenitis (OR-0.806, 95%CI-0.112-5.827) and last in duodenal ulcer (OR 0.271, 95% CI-0.084-0.879).

ELISA	Sensitivity (C.I.) *	Specificity (C.I.)	PPV (C.I.)	NPV (C.I.)	Accuracy
IgG	100 (78.9-100.0)	48.6 (46.8-48.6)	14.3 (11.3-14.3)	100 (96.3-100.0)	52.63
IgA	71.4 (48.7-87.6)	61.4 (59.2-63.0)	15.6 (10.7-19.2)	95.6 (92.0-98.1)	63.16

[Table/Fig-1]: Results of Novatec EIA- IgG and EIA- IgA compared with the results of biopsy based tests. *C.I. - Confidence interval

ELISA		Final result		Total
		Negative	Positive	
IgG/IgA	Neg/Neg	86	0	86
		100.0%	0%	100.0%
	Neg/Pos	16	0	16
		100.0%	0%	100.0%
	Pos/Neg	43	3	46
		93.5%	6.5%	100.0%
	Pos/Pos	65	15	80
		81.2%	18.8%	100.0%
Total		210	18	228
		92.1%	7.9%	100.0%

Chi-square value = 21.826, p-value <0.05, Significant
[a]combined results of RUT, Gram's staining, Histopathology and Culture

[Table/Fig-2]: Combined results of EIA-G update and EIA-A update compare with the results of the reference method[a]

Subjects with	IgG positive	IgG		IgA positive	IgA		Combined result of IgG and IgA
		OR*	95% C.I.*		OR*	95% C.I.*	
Reflux Esophagitis	57/94 (60.6%)	1.451	0.850-2.477	43/94 (45.7%)	1.289	0.756-2.197	39 (41.5%)
Gastritis	63/115 (54.8%)	0.962	0.570-1.621	46/115 (40%)	0.840	0.496-1.422	36 (31.3%)
Duodenitis	2/4 (50%)	0.806	0.112-5.827	2/4 (50%)	1.383	0.191-9.995	1 (25%)
Duodenal Ulcer	4/15 (26.7%)	0.271	0.084-0.879	5/15 (33.3%)	0.670	0.222-2.029	4 (26.7%)

[Table/Fig-3]: Correlation between various gastro duodenal diseases with anti-*H.pylori* IgG and anti -*H.pylori* IgA. *OR- odd ratio, *C.I. - confidence interval

IgA response was more common in duodenitis patients (OR-1.383, 95%CI-0.191-9.995) and reflux esophagitis patients (OR 1.289, 95% CI-0.756-2.197) and least in duodenal ulcer patients (OR 0.670, 95%CI-0.222-2.029). Even though the Overall IgG and IgA response out of 228 patients was highest in the gastritis patients (63 vs.46), followed by reflux esophagitis patients (57 vs. 43), while it was least in duodenitis and duodenal ulcer.

DISCUSSION

In view of the patchy distribution of *H.pylori*, all biopsy-based tests may theoretically fail to diagnose the infection. In contrast to biopsy-based methods, non-invasive tests assess the global presence of *H.pylori* in the stomach even when the bacteria are irregularly distributed on the gastric mucosa and it also obviates the need for endoscopy [9]. *H.pylori* infection provokes both local and systemic antibody responses. The systemic response typically comprises a transient rise in IgM, followed by a rise in specific IgA and IgG maintained throughout infection [10]. Almost all *H.pylori* infected individuals have elevated levels of specific IgG antibodies, but only in about two-third of cases does the IgA titer exceed the cut-off level [1].

The IgG, evaluated in our study, had a sensitivity and negative predictive values of 100% respectively, which is almost in accordance with other studies [11-13] [Table/Fig-4]. The high sensitivity observed permits the safe use of the test in epidemiologic surveys. Although we got very less specificity (108 samples were false positive), indicating that the ELISA must be validated for different populations. As it is proved earlier that sensitivity and specificity of serology depends on the gold standards used to compare the tests, the nature of the antigens employed and the value chosen for the cutoff and it is highly variable, ranging from 30%-100% [11]. Certainly other variables than this might be responsible for the differences observed between various studies, such as the severity and staging of peptic disease and the different strains of microorganism might also play a role [14].

Authors	Sensitivity	Specificity	PPV†	NPV††	Accuracy
Y Urita et al., 2004	94.8%	89%	91%	93.6%	92.1%
Sufi HZ Rahman et al., 2008	96.7%	42.8%	83.1%	81.8%	82.9%
Rosemary C She et al., 2009	87.6%	61%	22.8%	97.4%	64.2%
Present study	100%	48.6%	14.3%	100%	52.6%

[Table/Fig-4]: Comparison of IgG antibody parameters with different studies. †PPV- positive predictive values, ††NPV- negative predictive value

Our study also substantiates that the association between IgG and IgA will result in a marked improvement of the negative predictive value in comparison with the two assays alone but will not offer advantages in relation to positive predictive value .The 100% NPV of IgG makes it useful as a screening test and thus serves as an alternative to endoscopy.

In agreement with earlier reports [15-17] the results of the present study show that there is a significant association between detection of antibody and *H.pylori* infection. When both the IgG & IgA are negative *H.pylori* infection is also negative and the proportion of *H.pylori* infection is higher in patients who had both IgG and IgA positive result(p-value - 0.01, significant).

IgG response was more common in reflux esophagitis patients and then in gastritis [Table/Fig-3]. This data states that IgG response is associated with increase (1.451 times) risk of reflux esophagitis, followed by gastritis and duodenitis. Wyatt et al., [18] also suggested that the serological index of IgG antibodies against *H.pylori* is related to the severity of antral gastritis and the density of antral colonization.

The results of IgA ELISA are very well correlated with other studies [13, 14] [Table/Fig-5]. Although IgA ELISA showed lower sensitivity compare to IgG ELISA [Table/Fig-1] because most of the individuals (>90%) exhibit a predominant IgG immune response to infection with *H.pylori* [19]. And approximately 70% of these individuals also exhibit IgA antibodies. However some investigators [15, 20] have found that about 2% of the patient produces an IgA response in absence of IgG response.

Authors	Sensitivity	specificity	PPV†	NPV††	Accuracy
Angelo Locatelli et al., 2004	72%	65.9%	72%	67.4%	69.8%
Rosemary C She et al., 2009	63.4%	67.6%	17.6%	94.4%	67.2%
Present study	71.4%	61.4%	15.6%	95.6%	63.1%

[Table/Fig-5]: Comparison of IgA antibody parameters with different studies. †PPV- positive predictive values, ††NPV- negative predictive value

In our population we found 7% (16/228) of IgA- positive response and IgG –negative response. Jaskowski et al., [21] also showed higher frequency (7.2%) of IgA -positive and IgG -negative patients. Luthra et al., [19] also states that approximately 7% of infected individuals are positive for IgA antibodies but negative for IgG antibodies; the reason for this aberrant response remains unclear

IgA response was more common in Duodenitis patients and reflux esophagitis patients and least in duodenal ulcer patients. On the contrary Granberg et al., [15] establish the importance of IgA, as an increase risk factor of peptic ulcer disease and gastric cancer. Kosunen et al., [22] also showed the prevalence of IgA antibody highest in gastric ulcer patients, second highest in duodenal ulcer and chronic gastritis and are associated with the serious sequel of *H.pylori* infection.

CONCLUSION

EIA-G update is reliable and accurate test and because of its 100% sensitivity and negative predictive values, makes it useful screening test and thus serve as an alternative to endoscopy. Great care is to be taken not to underestimate the prevalence of *H.pylori* infection from the results of IgG serology in clinical practice. A positive finding of IgA antibody may be of significant clinical value in supporting diagnosis of infection especially if IgG serology is negative.

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