Validity of Salivary Polymerase Chain Reaction in Diagnosis of *Helicobacter pylori* Among Egyptian Patients

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Objective: *Helicobacter pylori* is highly endemic in Egypt. Salivary polymerase chain reaction (PCR) offers an easy and safe approach for disease detection as saliva contains an abundance of its biomolecules.

Aim of the Work: To evaluate the validity of salivary PCR as a quantitative method in diagnosis of *H. pylori*.

Methods: This study included 50 attendant patients of Gastrointestinal Endoscopy Unit, Faculty of Medicine, Cairo University, Egypt. They all proved histologically to have *H. pylori*–induced gastric and/or duodenal pathology. Another 50 patients negative for *H. pylori* were included as control group. All patients underwent stool antigen test and salivary PCR.

Results: Prevalence of *H. pylori* in clinically manifested Egyptian patients was 62.5%. The commonest endoscopic findings were gastric affection (90%), and third of cases (34%) showed definite ulcerative lesions. Salivary PCR test was significantly ($P < 0.001$) higher in *H. pylori* patients (mean, 10179.0 ± 20244.1 copies/dL) with wide range than in control group (mean, 99.2 ± 17.9 copies/dL), with sensitivity 100%, specificity 82%, and overall accuracy of 91%. Among the common complaints, it was significantly related to heartburn.

Conclusions: Salivary PCR proved to be a reliable diagnostic test, with sensitivity 100%, and accuracy reached 99% at cutoff level = 130 copies/dl (area under the curve was 0.998 at confidence interval = 0.993–1).

Key Words: *Helicobacter pylori*, salivary PCR, stool antigen test

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Helicobacter pylori is a ubiquitous infection, almost invariably transmitted oro-orally and feco-orally.[1] Its prevalence parallels that of gastric infection[2] and is the main etiological infectious agent causing peptic ulcer disease and gut cancer.[3] The criterion standard for the diagnosis of *H. pylori* infection is the invasive endoscopy with histology and culture of gastric biopsy.[4] However, noninvasive tools, including stool antigen test (SAT), are recommended as first-line diagnosis, for their high accuracy.[5,6] Stool antigen test is an easy reliable diagnostic test in *H. pylori* documenting infection and confirming eradication shortly after treatment.[7] Real-time (RT)-polymerase chain reaction (PCR), in gastric biopsies, is considered a reliable and sensitive diagnostic test in diagnosis of *H. pylori* infection,[8,9] including monitoring its eradication.[10] Moreover, it may be applied in diagnosis of genetic sequences responsible for antibiotic resistance.[9,11] The mouth is the first extragastric reservoir for *H. pylori*,[12] accordingly, salivary samples may be candidate media in diagnosing *H. pylori* by RT-PCR,[13,14] because the oral cavity is a potential reservoir for *H. pylori*, providing a favorable microaerobic environment.[14]

PATIENTS AND METHODS

This was a cohort study that included 50 adult patients who presented with various upper gastrointestinal symptoms from August 2014 till April 2015 in the Gastrointestinal Endoscopy Unit at Kasr Al-Ainy Hospital, Cairo University.

- Inclusion criteria: Adult patients, various gastrointestinal complaints, for example dyspepsia, heartburn, abdominal pain, hematemesis, loss of weight, and positive gastric pathology diagnosing *H. pylori.*
- Exclusion criteria: Patients receiving antipeptic ulcer therapy, for example, antacids, H2 receptor, and proton pump inhibitors or antibiotics. Peptic ulcer disease due to other etiology, for example, medications, immunologic, radiation, and so on. Gut neoplasm due to other etiology, for example, Crohn disease, and so on.

Another 50 adult patients complaining from various upper gastrointestinal symptoms and whose endoscopic pathology proved negative for *H. pylori*, served as the "Control group." They were age-matched and sex-matched with the positive cases.

This study was approved by the Ethical Committee of the department.

All included patients were subjected to: (1) Informed written consent. (2) Full history taking via a questionnaire with special emphasis on personal, occupational, special habits, drugs history, and indication of upper gastrointestinal endoscopy. (3) Full upper gastrointestinal endoscopic examination which was done using OLYMPUS CV-240 endoscope under conscious sedation using Midazolam 5 mg. (4) Histological examination for the detection of *H. Pylori* infection; 2 biopsies were taken from the prepyloric area. After hematoxylin-eosin and modified Giemsa staining, an experienced gastrointestinal pathologist. (5) *Helicobacter pylori* SAT detection was done using a commercially available kit *H. pylori* Ag Rapydtest which is a lateral flow chromatographic immunoassay for the qualitative detection of *H. pylori* antigen in human fecal specimen. It uses a colloid gold conjugated monoclonal anti–*H. pylori* antibody and another monoclonal anti–*H. pylori* antibody to specifically detect *H. pylori* antigen present in the infected patient's fecal specimen. About 1-g random stool specimen was collected in labeled sterile screw-cap leak proof container. No preservative substance was added to the stools. Watery, diarrheal specimens were not acceptable. Each test is composed of a nitrocellulose membrane strip containing a test band (T band, precoated with monoclonal anti–*H. pylori* antibody) and a control band (C band, precoated with goat antimouse IgG antibody). The test is read positive when both C and T bands developed and negative when only a C band. (6) *Helicobacter pylori* salivary PCR test; (a) Salivary samples collection was accessed from Molmeth (URL:http://www.molmeth.org/protocols/1BXQD00) as per the protocol derived from the World Health

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TABLE 1. Demographic Features of the Studied Patients

The Demographic Features		Results
Sex	Male/female	23 (46)/27 (54)
Age, y	Range	19–57
	Mean ± SD	42.0 ± 11.6
	Median (IQR)	47.0 (32.0–52.0)
Residence	Urban/rural	45 (90)/5 (10)
Medical history	Smoking	1 (2)
	Comorbidity (liver disease)	2 (4)
	Drug history	18 (36)

All demographic features, except age, are expressed in frequency (percentage).

Organization/International Agency for Research and Cancer guideline "Common Minimal Technical Standards and Protocols." Whole-saliva samples were obtained in the morning, patients were asked to only rinse their mouth out well (without drinking the water). Five minutes after this oral rinse, patients were asked to spit whole saliva in a sterile container. They were also asked not to cough up mucus during the saliva collection. About 5 mL of whole saliva was collected from each individual. and stored at −70°C until DNA extraction was done. (b) DNA was extracted from saliva samples by using QIAGEN DNA extraction kit (Germany) according to the instructions of the manufacturer. Briefly, 200 µL saliva, 25 µL QIAGEN Protease, and 200 µL buffer AL (lysis buffer), and 20 µL of proteinase K were added, mixed and incubated at 56°C for 10 minutes. After brief centrifugation, 250 µL of cold ethanol (96–100%) was added and mixed. The obtained mixture was applied to the QIAmp mini spin column and centrifuged at 8000 rpm for 1 minute. Pure DNA was eluted from the column using buffer AE (10 mM Tris-Cl; 0.5 mM ethylene diamineteraacetic acid (editic acid); pH 9.0) and stored at −20°C till further analysis. (c) Quantitative RT-PCR test using commercially available kit using SYBR-Green PCR master mix as described by O'Toole et al.[15] Real-time PCR measures a fluorescent signal that is proportional to the amount of amplified DNA. The most reliable point for quantification of template DNA is the cycle number at which the PCR product fluorescence becomes greater than a threshold. The fluorescence threshold represents the cycle number (Ct cycle) at which dye binding to PCR product generates a signal approximately 3 standards deviations above background. An arbitrary threshold level is inversely

TABLE (2-A). Comparison Between SAT and Salivary PCR Via X Square Test

Diagnostic Tests		Number (%)		*P*
		H. pylori Cases	Control Group	
SAT	Positive	41 (82)	4 (8)	<0.001
	Negative	9 (18)	46 (92)	
Salivary PCR	Positive	50 (100)	9 (18)	<0.001
	Negative	0	41 (82)	

proportional to the log of the initial amount of template DNA. Thus, the more starting template DNA, the fewer PCR cycles are required to reach the threshold.[16]

Quantitative real-time PCR assay amplification, data acquisition, and data analysis were carried out using quantitative real time PCR procedure on step 1 (Applied Biosystem Foster City, USA): to amplify *H. pylori* DNA, the real-time PCR used:

- –Double stranded DNA-specific dye SYBR Green I (Applied Biosystems).
- –The RT-PCR targeted the 26 KDa *Helicobacter* species-specific antigen gene,[16] the most widely applied and highly sensitive for not amplifying any other bacterial genes.[17]
- –The forward primer: 5′-TGGCGTGTCTATTGACAGCG AGC-3′.
- –The reverse primer: 5′-CCTGCTGGGCATACTTCACC ATG-3′) as previously described by Mikula et al.[18]
- –Positive control consisted of genomic DNA extracted from *H. pylori*.
- –Negative control was added in the form of distilled water to check the absence of contamination.

(d) Calculation of *H. pylori* level; to evaluate the detection limit of this reaction, serially diluted samples of *H. pylori* DNA ranging from 10^{-1} to 10^{6} femtogram (fg) [fg, a unit of mass = 10^{-15} g] were used as a template. Each sample was tested and the final threshold cycle (Ct), that is, the point at which sample fluorescence rises above the background level was determined. A standard curve, constructed by plotting the log of the initial DNA concentration versus the Ct value was used to determine *H. pylori* DNA concentrations.[17] The lowest *H. pylori* DNA concentration detected was 10 fg, equivalent to 5 bacterial cells.

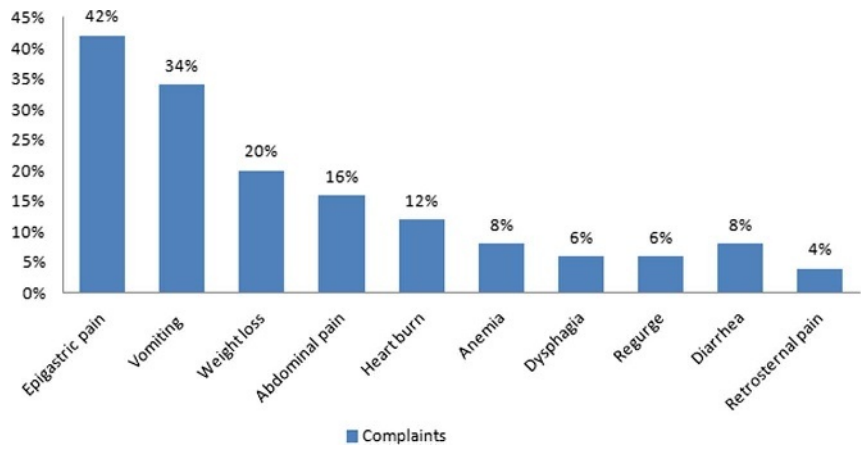

FIGURE 1. Complaints of the *H. pylori* studied patients.

TABLE (2-B). Comparison Between the 2 Specific *H. pylori* Studied Tests

The Studied *H. pylori* Tests	Sensitivity	Specificity	PPV	NPV	Accuracy
SAT	82.0%	92.0%	91.1%	83.6%	87.0%
Salivary PCR	100.0%	82.0%	84.7%	100.0%	91.0%

PPV, positive predictive value; NPV, negative predictive value.

Statistical Analysis

Precoded data were entered into the Statistical Package of Social Science Software program, version 21 (SPSS) to be statistically analyzed. Data were summarized using range, mean, standard deviation, and median with interquartile range (IQR) for quantitative variables and frequency and percentage for qualitative ones. Comparison between groups was performed using Mann Whitney test for quantitative variables and χ^2 with Fisher exact tests for qualitative ones. Receiver operating characteristic curve was plotted to determine the optimal sensitivity and specificity of HP salivary PCR among all the studied patients. It is considered a significant and reliable discriminator when area under the curve is 0.9, and suggestive for discrimination when 0.7 to 0.89. *P* values less than 0.05 were considered statistically significant and less than 0.01 were considered highly significant.

RESULTS

This study was conducted on patients attending the Endoscopy Unit at Kasr Al-Ainy Hospital, Cairo University in the period from August 2014 till April 2015. The study included 50 patients proved histologically to have *H. pylori*–induced gastric and/or duodenal pathology. These patients were recruited out of 80 patients presenting with endoscopic signs of peptic ulcer disease or various degrees of gastritis and/or duodenitis, that prevalence of *H. pylori* was 62.5%. The demographic features of the studied patients are shown in Table 1. The studied patients were mainly middle aged with sex comparably presented, and mostly came from urban areas.

The studied patients' complaints are illustrated in Figure 1, commonest of them were epigastric pain (42%) followed by vomiting (34%).

The endoscopic examination revealed that gastric affection was commoner, and double the duodenal one (45/50; 90%, and 22/50; 44%, respectively). In addition, inflammatory reaction was common (34/45; 75.6%, and 16/22; 72.7%, respectively). Interestingly, none of the patients had malignant affection.

Stool antigen test in comparison to salivary PCR test in all of the studied patients are shown in Tables 2-A, 2-B, 2-C. Tables 2-A and 2-B, via χ^2 test, showed sensitivity, specificity, positive predictive value, negative predictive value, and accuracy, whereas Table 2-C showed the values recorded by test of interest of the study, that is, salivary PCR test. Our study demonstrated that the agreement between HP salivary PCR and its SAT was 82%, and the former test level was higher in SAT positive (median, 4890.0 copies/dL; IQR, 1585.5–10891) than in SAT negative (median, 1938.0 IQR, 697.5–8682 copies/dL) but without any statistical significance.

On the other hand, it did not show a significant difference in terms of SAT ($P = 0.2$). Its median and IQRs in positive and negative SAT were 4890.0 (1585.5–10891.0) and 1938.0 (697.5–8682.0) copies/dL, respectively.

The relation between both SAT and salivary PCR tests in HP patients' complaints and endoscopic findings are shown in Table 3. The uncommon complaints were not included for being not reliable statistically.

The receiver operating characteristic of HP salivary PCR test is illustrated in Figure 2. Area under the curve was 0.998 at confidence interval = 0.993–1.

DISCUSSION

Helicobacter pylori causes wide range of gastroduodenal affection, that is, gastritis and/or duodenitis and peptic ulcer disease,[19] and a definite carcinogen leading to gastric cancer.[19,20] It is highly endemic in Egypt, where its prevalence reaches 90% among adults,[21] even among healthy asymptomatic population.[22] Invasive diagnostic tests via endoscopy are indicated in certain symptoms, for example, weight loss, anorexia, hematemesis, recent onset of dyspepsia in rather old patient, and failure of previous eradication treatment.[23] *Helicobacter pylori* also inhabits the oral cavity and likely to transmit its infection,[24] for the proved close relation between *H. pylori* infection in the oral cavity and the stomach.[12]

This study aimed at evaluating the diagnostic role of salivary RT-PCR for *H. pylori*. The present study showed that out of 80 patients who underwent endoscopy, only 50 patients proved histologically to have *H. pylori*–inducing peptic ulcer disease. Thus, its incidence reached 62.5%. It is worth noting that this figure was reported among the clinically manifested *H. pylori*, not among the whole spectrum of affection, yet, *H. pylori* prevalence shows recently a trend to decrease.[25]

In this study, patients were mainly middle aged (42.0 ± 11.6 years) with comparable gender presentation, that is, 46% men versus 54% women. Infection generally takes place during childhood, then inflammatory changes progress and go on throughout life.[3]

In this study, more than half of the patients (58%) presented with dyspeptic symptoms; either limited to epigastric pain (42%) or diffuse abdominal pain (16%). This agreed with Bazzoli et al[26] and Hu et al[27] who found dyspepsia to be strongly related to *H. pylori* infection and its remarkable resolution after *H. pylori* eradication.[27]

On the other hand, the commonest endoscopic finding was gastric affection (90%), followed by duodenal affection (44%). All of the patients had nonulcer inflammatory lesions, for the fact that inflammation takes place even if *H. pylori* did not penetrate the gastric epithelium.[28] However, this was higher than other studies like Rudelli et al's.[29] Third of the cases (17/50; 34%) showed definite ulcer affection. This figure may be considered low for the proven strong relation between *H. pylori* and peptic ulcers.[25] Meanwhile, almost 2/3 of the ulcerative lesions were gastric (11/17;

TABLE (2-C). Salivary PCR Levels Among the Studied Patients

Salivary PCR, copies/dL	The Studied *H. pylori* Patients	The Control Group	*P*
Range	98.0–121530.0	68.0–120.0	<0.001
Mean ± SD	10179.0 ± 20244.1	99.2 ± 17.9	
Median (IQR)	4020.5 (1217.0–10582.0)	102.0 (93.0–112.0)	

TABLE 3. The Relation Between Both SAT and Salivary PCR Tests in *H. pylori* Patients' Complaints and Endoscopic Findings

The Studied Parameter		Positive / Negative	SAT N (%) Positive (n = 41)	SAT N (%) Negative (n = 9)	*P*	Salivary PCR Median Value ± IQR, copies/dL	*P*
The patients' main complaints	Epigastric pain	(n = 21)	13 (31.7)	8 (88.9)	0.002	2485.0 (1111.0–9496.0)	0.4
		(n = 29)	28 (68.3)	1 (11.1)		6542.0 (1245.5–11780.0)	
	Vomiting	(n = 17)	15 (36.6)	2 (22.2)	0.7	2850.0 (1585.5–10561.0)	0.9
		(n = 33)	26 (63.4)	7 (77.8)		6325.0 (1157.5–10746.0)	
	Weight loss	(n = 10)	9 (22.0)	1 (11.1)	0.7	2211.50 (1198.5–11370.0)	0.4
		(n = 40)	32 (78.0)	8 (88.9)		5607.5 (1353.3–10618.0)	
	Abdominal pain	(n = 8)	7 (17.1)	1 (11.1)	1.0	6472.0 (694.5–24414.8)	0.7
		(n = 42)	34 (82.9)	8 (88.9)		4020.5 (1259.8–10550.5)	
	Heart burn	(n = 6)	3 (7.3)	3 (33.3)	0.006	9029.5 (2653.0–13844.0)	0.3
		(n = 44)	38 (92.7)	6 (66.7)		3317.5 (1183.3–10505.3)	
The patients' main endoscopic findings	Gastritis	(n = 34)	27 (65.9)	7 (77.8)	0.7	4020.5 (1259.8–10363.8)	0.9
		(n = 16)	14 (34.1)	2 (22.2)		5386.5 (694.5–12706.8)	
	Duodenitis	(n = 16)	13 (31.7)	3 (33.3)	1.0	2491.0 (923.3–10825.0)	0.6
		(n = 34)	28 (68.3)	6 (66.7)		4573.0 (1741.3–10594.0)	
	Gastric ulcer	(n = 11)	11 (26.8)	0 (0)	0.2	3254.0 (1143.0–16920.0)	0.7
		(n = 39)	30 (73.2)	9 (100)		4256.0 (1217.0–10582.0)	
	Duodenal ulcer	(n = 6)	4 (9.8)	2 (22.2)	0.3	6204.5 (454.8–11290.3)	0.9
		(n = 44)	37 (90.2)	7 (77.8)		3519.5 (1231.3–10607.5)	

65%). whereas one third (6/17; 35%) showed duodenal ulcer. This was opposite to many previous studies where duodenal affection was evidently more dominant than gastric one.[27–29]

Conversely and interestingly, none of the patients had either complicated peptic ulcer disease or neoplastic growth, although *H. pylori* is known as a definite biologic carcinogen,[30] including mucosa associated lymphoid tissue lymphoma.[31,32] This may be explained by the finding that the more severe manifestations of the infection usually occur later in life, and in a minority of infected subjects.[3] Also, sex and pepsinogen status play role as independent predictors for carcinogenesis.[33]

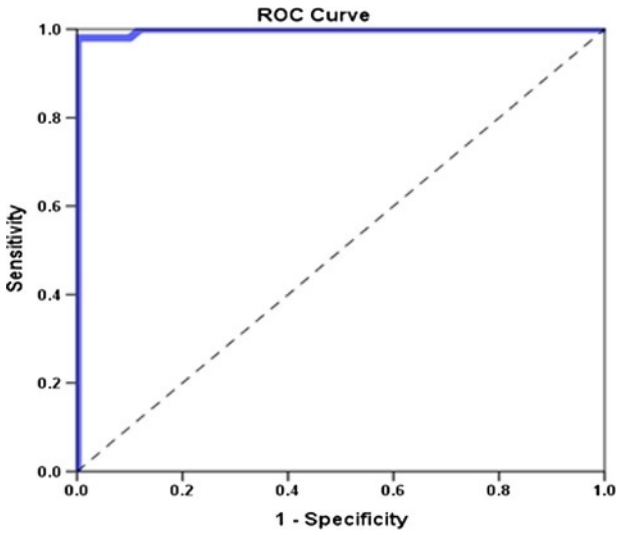

FIGURE 2. ROC of *H. pylori*-Salivary PCR among all patients (PPV = 100%, NPV = 98%). ROC, receiver operating characteristic. PPV, positive predictive value; NPV, negative predictive value.

Stool antigen test is a reliable simple noninvasive highly sensitive and specific diagnostic test for the qualitative detection of *H. pylori*, even for monitoring therapy.[34] However, in this study, sensitivity of SAT was 82% despite using the conjugated monoclonal anti–*H. pylori* antibody that specifically detect *H. pylori* antigen. This was lower than previous studies, that is, 86%,[35] 88.9%,[36] 94.1%,[7] 95%,[21] and 98.2%.[37] The excellent sensitivity results were achieved by the monoclonal test,[35] less sensitivity was reported in the polyclonal test was 88.3%[38]; although specificity was 92%, which was also lower than that recorded before, for example, 94%.[21]

There was a statistically significant difference regarding SAT in relation to epigastric pain as a complaint of the studied patients. However it did not show any significant relation with any of the endoscopic findings.

In this study, the sensitivity of *H. pylori* salivary PCR was recorded to be 100%. This matched with previous studies like Tiwar et al and Saxena et al,[39,40] who found comparable sensitivity of salivary PCR of *H. pylori* to the gastric biopsy. It was higher than a previously reported serology technique (81%)[41] and higher than our SAT. Moreover, it was also higher than that previously found among Egyptian dyspeptic patients 85%.[42] *Helicobacter pylori* detection in saliva ranges from 10% to 54.1% of infected patients confirming it as a favorable reservoir.[43,44] Also, it is considered a reliable, accurate, and acceptable test, particularly among adults with intellectual disability.[45] However, this was contrary to the conclusion stated by Rossi-Aguiar's et al.[46] On the other hand, specificity was lower than SAT when studied at qualitative level, but it increased to 100%, and its accuracy reached 99% at cutoff level 130 copies/dL. The recorded sensitivity and specificity in this study was higher than that recorded previously in Egypt (75% and 100%, respectively),[42] which may be attributed to the fact that it was limited to dyspeptic patients only.

In this study, the salivary PCR levels among the HP patients recorded a very wide range of values with a mean of 10,179.0 ± 20,244.1 copies/dL and showed no significant

relation to any of the presenting complaint nor with the endoscopic findings. This was similar to the study by Sayed et al.[42] On the contrary, levels were significantly lower in the control group and with very narrow range.

CONCLUSIONS

Salivary RT-PCR test is highly sensitive in diagnosing *H. pylori*, recording very wide range of recorded values, although it did not record a significant differentiating value related to either the presenting symptom or the endoscopic finding. Extensive study with more patients is recommended to identify more accurate results.

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