DNA detection of *Helicobacter pylori* in saliva of patients with low salivary pH

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Abstract

Background and objective: One of the well-organized diagnostic methods of *Helicobacter pylori* (*H. pylori)* infection is bacterial DNA detection in saliva using polymerase chain reaction (PCR). However, a wide range of factors is present in human saliva that could hinder detection efficiency. One of these factors seems to be the pH of saliva, which is investigated in this study as to whether its variation could have any effects on *H. pylori* DNA presence in the saliva of infected patients.

Methods: Saliva samples of 89 *H. pylori*-infected Patients were collected and measured for pH levels. The DNA of *H. pylori* was extracted from the saliva of patients and then quantified directly by Real-time PCR. One Way ANOVA and Linear Regression tests were performed to determine the effect of pH on the detection of *H. pylori* DNA in the saliva.

Results: 58% of samples were PCR positive (17% high, 19% medium, and 22% low titer), and no DNA was detected in 42% samples. There was no significant association between the age of patients and the detection of DNA (*P* = 0.98). A statistically significant difference in pH level was found between negative and positive PCR samples. Calculation of linear regression of DNA copy numbers and independently increasing pH showed $R^2 = 0.041$.

Conclusion: The ability to detect *H. Pylori* DNA was generally confined to pH of 5.5 to 7.5 with an apparently reduced detectability at above neutral pH of saliva. However, pH variation from 5.5 to 7.5 did not determine the levels of detected DNA of *H. pylori* in saliva.

Keywords: *H. pylori*; Gastric ulcer; Real-Time PCR; Saliva; Salivary pH.

Introduction

H. pylori are gram-negative spiral bacteria that reside in the gastrointestinal tract of human. They have a narrow host range and habitually establish a lifelong infection suggesting a robust adaptation to its habitat. The bacteria lack a number of biosynthetic pathways for several amino acids, which make limited *in vitro* growth.[1] They are responsible for gastric ulcer and a well-recognized risk factor for gastric cancer.[2] In this regard, gastric cancer is considered as the third leading cancer-related cause of death globally, with *H. pylori* infection being responsible for nearly 75% of all non-cardia gastric cancer cases.[3,4] Annually, million deaths worldwide occur due to gastric and peptic cancers making it a globally important health issue.[5] In view of the fact that the main route of transmission of *H. pylori* is fecal-oral, the higher levels of infection are associated with low levels of sanitation and socioeconomic status.[6] A number of studies have demonstrated the association of gastroesophageal infections with the presence of *H. pylori* in the oral cavity.[7,8] Thus, failure to eradicate *H. pylori* from the oral cavity might result in gastrointestinal reinfection.[9,10] Despite the eradication of *H. pylori* from the stomach, early reinfection of the stomach can be promoted due to the survival of bacteria in the oral cavity. Such a function of the oral cavity in serving as

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a sanctuary for *H. pylori* could reduce the efficacy of eradication therapy.[11] Poor salivary secretion can possibly lead to the dental plaque deposition which can also provide a favorable environment for the growth of bacteria such as *H. pylori*.[12] Several types of salivary glands secrete saliva, each with different compositions and properties. Accordingly, the secretions from various glands considerably differ in composition and are affected by different stimuli, diet, time of day, age, gender, health status, and various pharmacological substances. Whole saliva is a clear, slightly acidic (pH 6 to 7), mixed fluid containing mucosal transudations, gingival crevicular fluid, expectorated nasal and bronchial secretions, desquamated epithelial cells, debris from food, bacterial products, viruses, and fungi[13]. Saliva has often been proposed as a diagnostic tool specifically promoted by the current technological advancement such as PCR, Western blotting assay and Enzyme-linked Immunosorbent Assay(ELISA).[14] Various diagnostic tools have been developed to detect *H. pylori*, including invasive (e.g., endoscopic based) and non-invasive techniques. Among non-invasive diagnostic methods, the most commonly applied are stool antigen test, urea breath test, serological test, and molecular techniques.[15] Saliva is often taken for molecular detection of *H. pylori* at the DNA level.[16] In that sense, the salivary components could affect the survival of bacteria and, more pertinently, the integrity of DNA used for PCR. Moreover, the pH of saliva could implement its own impact on the presence of *H. pylori* in the oral cavity. One instant is likely to be the function of salivary nitrite on the killing of *H. pylori*.[17] Thus, this study was performed with the aim to define the association of salivary pH with the presence of *H. pylori* DNA and further with the growth of *H. pylori* in saliva.

Methods

Study design
This prospective study was performed on 89 patients admitted to Rizgary Teaching Hospital, whose gastric biopsy showed positive growth for *H. pylori*. All work and protocols in this research were approved by the Ethics Committee in the College of Medicine at Hawler Medical University (Paper Code: 5 issued on 20-12-2018). The handling of the biological specimen was done according to the Declaration of Helsinki criteria.[18] Samples of saliva were collected from June to November 2018 from Rizgary Teaching Hospital. The exclusion criteria were: antibiotics within the previous two weeks, age over 80 years, and severe concomitant diseases.

Sample collection
The patients were informed about the procedure, and information on gender and age was recorded (Table 1) before sampling. 5mL saliva was collected and placed into a sterile polystyrene tube with the cap was firmly closed according to the previous work by Cellini et al.[19] The specimens were immediately transferred within less than 2 hours at $10^0C – 15^0C$ to Genome Diagnosis Lab (GDL).

Saliva analysis
The saliva samples were measured for their pH using Professional Benchtop pH meter BP3001 (Trans Instruments, Petro Centre, Singapore). DNA extraction was done using Bacterial DNA Preparation kit (Jena Bio Science, Thuringia, Germany). Briefly, saliva was vortexed for 10 seconds to get a homogenous suspension then 500µL was transferred into a 1.5mL cap tube. After centrifugation at 1000g for 1 minute, the supernatant was discarded, and the pellet was resuspended in 300µL Lysis Buffer, 2µL RNase A, and 8µL of Proteinase K then incubated at 60^0C for 10 minutes. The solution was then added to a spin column and washed twice with washing solution via centrifugation. DNA was recovered by adding 50µL DNase-RNase free water to the column and centrifuging for 2 minutes at 1000g. The DNA was measured for quantity and purity via Nanophotometer (IMPLEN, CA, USA).

Real-Time PCR

Real-time PCR was performed to bacteria extractions by RealLine™ Helicobacter pylori Str-Format kit (VBD3798, BIORON, Ludwigshafen, Germany). Using Eco Real-Time PCR machine (Illumina, CA, USA) 50µL of the isolated DNA solution was added to the ready master mix tube. The tubes were placed into the Real-Time PCR system programmed as 50^0C for 2 min initiation and 95^0C for 2 min initiation. Then it was followed by 50 cycles of 94^0C for 10 seconds and 60^0C for 20 seconds. The fluorescence intensity was measured at 60^0C. FAM channel was set to detect Internal Control amplification, and ROX channel was set to collect *H. pylori* DNA amplification signals. Positive and negative controls were applied according to the kit's instruction manual. Relative quantification of DNA (semi-quantification) was performed by measuring the difference between Ct (cycle threshold) values of positive control and the tested sample using $2^{-\Delta\Delta Ct}$ method.[20] The bacterial DNA load values were divided into three groups based on the mean concentrations (i.e., 45000copies / mL) of positive control. In this regards, the relative concentrations less than 10000 copies / mL were assigned as "Low", from 10000 to100000 copies/mL were named as "Medium", and concentrations above 100000 copies / mL were regarded as "High".

Data analysis

The Data analysis was done by GraphPad Prism v6. A *P* value of 0.05 was considered as statistically significant. Chi Square, Student t-test, and One Way ANOVA tests were applied to find out the statistically significant differences between studied groups.

Results

The patients' age was arranged as 10 years intervals with the number and percentage of each age group based on gender distribution (Table 1).

Detection of *H. pylori* DNA in relation to gender

There were 52 detected *H. pylori* DNA samples, of which 32 (61%) were male, and 20 (38.46%) were female (Table 2).

Table 1: Age and gender distribution of patients.

Age (years)	Female No. (%)	Male No. (%)	Total No. (%)
20 – 29	12 (31.57)	18 (35.29)	30(33.70)
30 – 39	15 (39.47)	16 (31.37)	31(34.83)
40 – 49	9 (23.68)	13 (25.49)	22(24.72)
≥ 50	2 (5.26)	4 (7.84)	6(6.75)
Total	**38 (100.0)**	**51 (100.0)**	**89 (100)**

Table 2: Detected and not detected DNA according to gender.

Gender	Detected No. (%)	Not Detected No. (%)	Total No. (%)	P value
Male	32 (62.7)	19 (37.3)	51 (100)	**0.338**
Female	20 (52. 6)	18 (47.4)	38 (100)	
Total	**52 (58.4)**	**37 (41.6)**	**89 (100)**	

Detection of H. pylori DNA in relation to age

The associations of the age of patients with the presence of *H. pylori* DNA in their saliva samples were investigated. The efficiency of DNA detection was greater at the age of 20 to 40 years, and it was found to be equal at older ages. Chi-square test was applied to compare the age groups regarding their level of DNA detection (Figure 1A). Nevertheless, taking the overall effect of age into account, there was no statistically significant difference between detection and no detection of *H. pylori* DNA in saliva at different ages. The t-test of two independent samples was performed to compare the overall detection level in all ages (Figure 1B).

Measurement of detected H. pylori DNA

H. pylori DNA in the saliva samples of patient was detected and quantified using Real-Time PCR method, as shown in Figure 2.
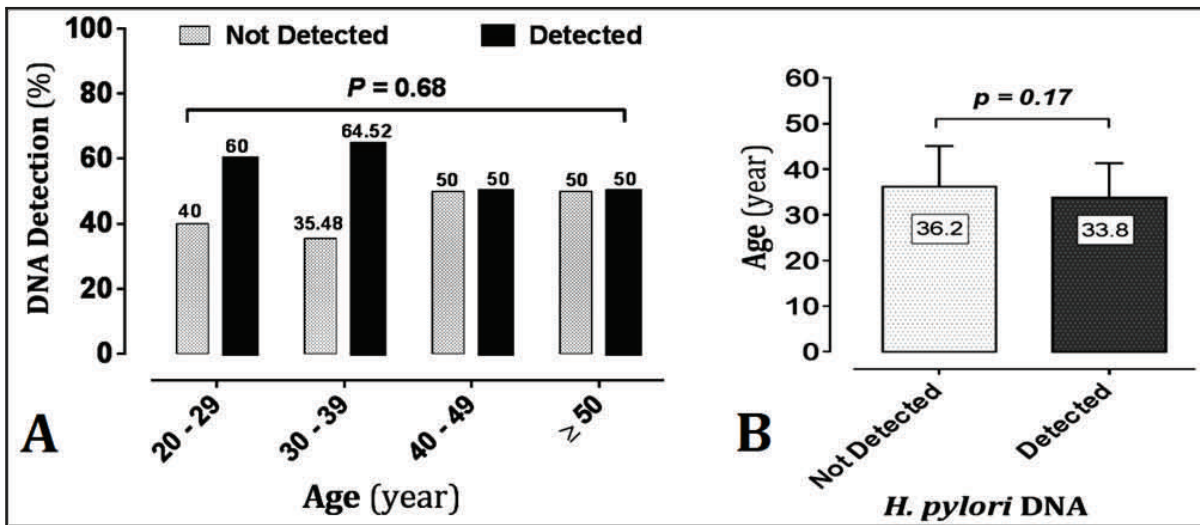
Figure 1: *H. pylori* DNA detection in the saliva is generally higher at younger ages. Error Bars = Standard Deviation
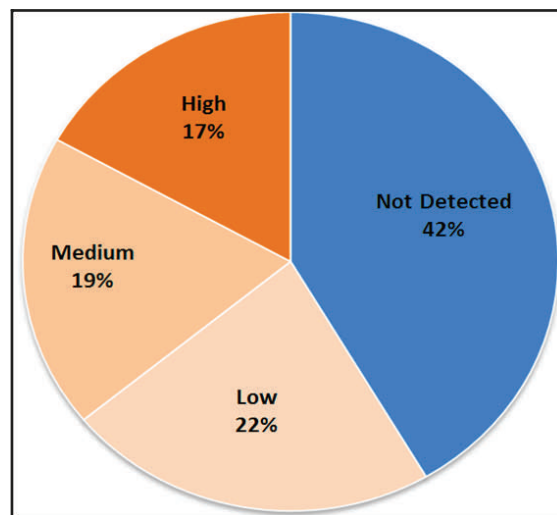
Figure 2: More than half of saliva samples were detected with *H. pylori* DNA. The samples with DNA detection were grouped into low, medium and high according to the quantification.

Association of bacterial DNA load with salivary pH

Detection of *H. pylori* DNA in the saliva and its association with the pH levels of saliva is shown in Table 3. A statistically significant difference was inferred among "High," "Medium," "Low," and "Not Detected" groups after ANOVA test. To explore further, the significant difference was only attributed to the "Not Detected" with other groups using Fisher's Least Significant Difference test.

Association of bacterial DNA load with salivary pH

The levels of *H. pylori* DNA copy number with increasing pH of saliva is shown in Figure 3. The relative concentration (copies / mL) of *H. pylori* DNA in saliva of patients was plotted, and the linear regression of concentration as to the independently increasing pH levels was measured.

Table 3: Association of salivary pH with the level of DNA detection.

DNA Detection	pH	
	Mean	**±SD**
High	6.17	0.66
Medium	6.30	0.62
Low	6.54	0.80
Not Detected	7.14	1.02
One Way ANOVA *(P value)*	0.0026	

Least Significant Difference:

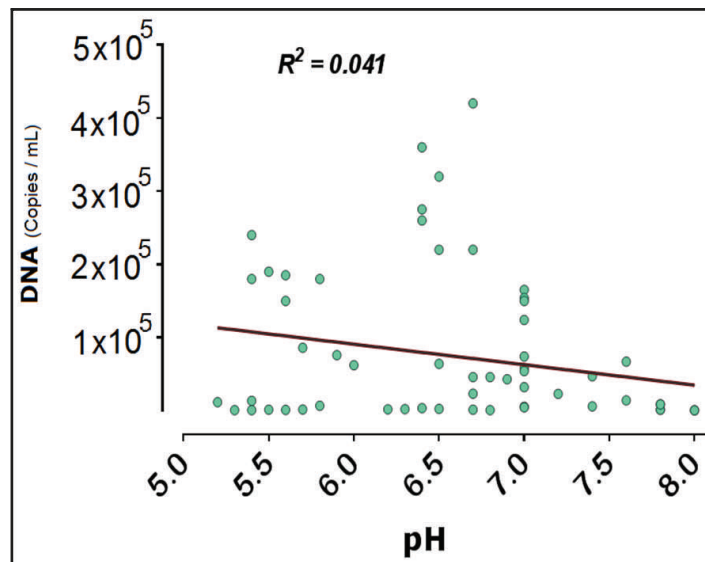|	
Not Detected vs. High:	*P* <0.0001
Not Detected vs. Medium:	*P* <0.0001
Not Detected vs. Low:	*P* <0.0001
High vs. Medium:	*P* = 0.0732
High vs. Low:	*P* = 0.6838
Medium vs. Low:	*P* = 0.1445

Figure 3: A scatter diagram of bacterial DNA load at increasing pH of saliva. *P* value = 0.07 and r = -0.23 **Line:** Best fit line from Linear Regression.

Discussion

H. pylori has been co-evolved with humans since the beginning of human history,[21] causing an infection that is considered one of the most prevalent chronic bacterial diseases worldwide.[22] A number of survival strategies developed throughout this association to persist the challenging environments in the human body. Indeed, the survival of *H. pylori* relies on its ability to circumvent the antimicrobial effects of stomach acid.[23] Nevertheless, the main reservoir of *H. pylori*, as suggested by the recent studies, is identified to be in the oral cavity.[24] Studies propose oral spread of bacteria via saliva to be one of the primary modes of transmission.[25] In this regard, detection of *H. pylori* DNA in saliva using PCR in this study appears to be an efficient way and to detect infection which has been applied by other studies.[26-28] Although a meta-analysis of relevant studies imply that gastric infection with *H. pylori* doubles the likelihood of finding *H. pylori* in the oral cavity,[29] it is also possible that the stomach colonization occurs independently of *H. pylori* detection in the mouth.[19] There might be many elements potentially involved, including age and gender of the infected individual as well as components of saliva. In view of that, the scheme of data shows a higher incidence of salivary *H. pylori* DNA in males than females (Table 2). Nevertheless, our investigation to find the possible link between age and detection of *H. pylori* DNA (Figure 1) revealed no significant effect though the younger ages were found with slightly higher DNA detection levels (Figure 1A). Having considered the age, it is well known that human saliva has miscellaneous substances and a variety of anti-bacterial mechanisms.[25] In an attempt to address the central concern of this study, relative quantification of DNA and relevant statistical analyses were applied to find out the impact of variation in salivary pH on *H. pylori* DNA detection. Thus, the 58% detection capacity (Figure 2) is comparable with similar studies in which the range of

detection varies from 10%[30] to 87.5%[31] and its similar to 51%.[16] It is well known that PCR has the highest efficiency in detection of *H. pylori* infection compared to routine tests such as culture[26,32] and rapid urease test (RUT). In reference 13, for instance, the odd ratio = 5.51 for PCR and odd ratio = 2.2 for RUT.[33] However, the inability to detect DNA in 42% of samples in this study seems to be inherent in other similar studies which can be attributed to the specific characteristics of saliva. The analyses show that increasing pH is statistically associated with negative PCR results (Table 3). Nonetheless, changes in pH were found to have no significant impact on the detection level (Figure 3). Such findings overall suggest the presence of mechanisms in saliva that might influence the salivary pH to exert anti-bacterial effects. Therefore, it is important to notice that despite the ability of *H. pylori* to tolerate the extremely acidic environment of stomach.[34] The bacteria face a relatively restricted growth in saliva, which is reasonably in agreement with the findings of this study. One conceivable reason can be the colonization of various microorganisms, particularly the fast-growing bacterial species in the oral cavity.[19] This can competitively inhibit the growth or often ends in entering *H. pylori* into a viable but not culturable (VBNC) state that limits the spread of *H. pylori* into the saliva.[35] The diversity in nitrite concentration of human saliva, which varies depending on dietary intake from 0.05 to 1.0 mmol/L[17] can be another reason for such discrepancy. Nitric oxide that has been recognized for its anti-bacterial function is a derivative of nitrate. In their study, Takahama and colleagues have observed that under acidic conditions, nitrate is protonated to nitrous acid, which can later be transformed into nitric oxide via reduction and self-decomposition. Thus, decreasing the pH of saliva increases the formation of nitric oxide depending on the nitrate level of saliva. Consequently, alteration in nitrate

levels in saliva could potentially change the survival rate of *H. pylori* and, in the meantime, changes the pH of saliva.[32] Taking into account the mentioned strategies and mechanisms that could possibly explain such a pattern of salivary pH on the detection of *H. pylori* DNA, the precise means upon which this effect can be explained has yet not been fully elucidated.

Conclusion

One of the most robust techniques to detect *H. pylori* in the saliva is Real-time PCR, which has been used in this study to investigate the association of salivary pH to bacterial DNA detection capacity. In this regard, pH of 5.5 to 7.5 was found to be more associated with the detection ability by PCR. However, the quantification of DNA showed no correlation between the increasing DNA quantities with the variation of pH within this range. Hence, the authors of this study recommend further studies on the link between salivary pH and *H. pylori* DNA detection with regard to a more detailed investigation of factors that can influence the presence of bacteria in saliva.

Competing interests

The authors declare no competing interests.

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