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Research Article

APPLICATION OF POLYMERASE CHAIN REACTION FOR DETERMINATION OF PREVALENCE OF HELICOBACTER PYLORI IN SALIVARY SAMPLES OF ASYMPTOMATIC SUBJECTS

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ABSTRACT

Objective: The present study was designed to determine the prevalence of *H. pylori* infection using polymerase chain reaction in asymptomatic human subjects.

Methods: The detection of genes from salivary samples by application of polymerase chain reaction confirmed *H. pylori* infection. The presence and absence of infection was statistically compared with various socio demographic factors. Relation of *H. pylori* infection with factors like gender, age, smoking, alcohol, NSAID use, food habit, marital status, water source, urban and rural residence, socioeconomic status was evaluated.

Results and Discussion: Prevalence of *H. pylori* infection was found to be instigated with factors like smoking, frequent NSAID use, rural residence, consumption of raw municipal water, low socioeconomic status whereas high socioeconomic class and consumption of alcohol reduce the chance of acquiring *H. pylori*. Gender and marital status were found to be not associated with the spread of infection.

Conclusion: Prevalence of *H. pylori* in the asymptomatic subjects varies with the different sociodemographic factors and needs to be checked by taking relevant measures.

Abbreviations: OR Odds ratio, CI Confidence interval, CTAB cetyl trimethyl ammonium bromide, PCR Polymerase chain reaction, *H. pylori* Helicobacter pylori

Keywords: PCR, *H. Pylori*, Prevalence.

INTRODUCTION

In India, the reported incidence of *H. pylori* infection is 85 % .[1,2] Inadequate data is available on *H. pylori* prevalence in Indian subjects and there is no documented proof of the present population of subjects who suffer from *H. pylori* infection in India. 16sr RNA gene represents a highly conserved region of *H. pylori* genome. This gene can be amplified using polymerase chain reaction and serve as a tool for the detection of *H. pylori* infection. Pathology laboratories in Pune do not use PCR to detect *H. pylori* infection. The objective of the present investigation was to detect the infection status in the asymptomatic subjects by PCR and to investigate the effect of the demographic factors like smoking, alcohol, age, gender, socio economic status, urban or rural residence, NSAID use with *H. pylori* infection.

MATERIALS AND METHODS

All the chemicals for DNA extraction were procured from S.D. Fine chemicals, India. The reagents for PCR, gel preparation, and visualization were purchased from Vivantis India, Thane. The forward and reverse primer for *16S rRNA* gene was synthesized at Ocimum Biosolutions, Hyderabad, India. Gel electrophoresis unit (Bangalore genie, Bangalore) was used to perform gel electrophoresis and Gel documentation unit (Alpha Innotech Inc. USA) was used to visualize and capture the gel image.

Human ethics committee approval

The study protocol was approved by Institutional human ethics committee of Bharati Medical College Bharati Vidyapeeth Deemed University, Pune.

Sample collection

A total of 1050 healthy subjects were included in the present study. Subjects with any of the following co-morbid conditions were excluded: chronic hepatic and renal disease, presence of malignancy, diabetes mellitus, and history of triple drug regimen or *H.pylori* eradication therapy. All subjects were inquired about symptoms like abdominal pain, bloating, belching, post prandial fullness, malena, hematemesis, early satiety and burning sensation in the chest suggestive of acid peptic disease. All individuals affirmative of these symptoms were excluded from the study. None of the participants had symptoms suggestive of acid peptic diseases. All individuals signed an informed consent in order to be included in the study. The study population consisted of men and women of more than 18 years of age. Saliva samples were collected by visiting homes, colleges and villages during the months of January to June 2010. Unstimulated saliva in the volume of 1.5 ml was collected in sterile container and stored at -80^0 C until processed. Approximately 1.5 ml of non-stimulated salivary flow was collected in a presterelised 2-mL microtube [3]. Thereafter, saliva was homogenized by vigorous shaking with the use of a vortex mixer and clarified by centrifugation (10,000 g, 4^0 C, 4 min)

Collection of data

The questionnaire was available for data collection that included the age, gender, and history of cigarette smoking, alcohol consumption, and NSAID use and water source level of the adult study subjects from urban and rural population in Pune region. The socioeconomic status was evaluated using a validated questionnaire [4]. All the subjects who consumed NSAIDs more than 10 day per month were considered as NSAID users [5].

Preparation of genomic DNA for PCR

DNA isolation from salivary samples was performed according to C-TAB method mentioned by [6]. Amplification of the DNA template was carried out using primers HP1 HP2 and HP3 [7]. 16S rRNA (480 base pair fragment) was amplified and this product was subsequently amplified to produce a 109 base pair fragment in a thermal cycler. The products were analyzed by agarose gel electrophoresis and the image of the gel was captured using gel documentation. A standard *H. pylori* strain (ATCC 26695) was used as positive control and double-distilled water as negative control in each PCR amplification.

Primer Sequences used in the study are as follows

HP1 5'- CTGGAGAGACTAAGCCCTCC-3'
HP2 5'- ATTACTGACGCTGATTGTGC-3'
HP3 5'- AGGATGAAGGTTTAAGGATT-3'

Statistical Methods

Statistical analysis was carried out to examine the associations between the various study variables with saliva PCR positivity for *H. pylori* using Fischer exact test. SPSS (version 17) (SPSS Inc., Chicago, IL,USA) was used to determine Odds ratio, 95% confidence interval of OR, Relative risk and 95% confidence interval of OR (Greenberg et al. 1996).

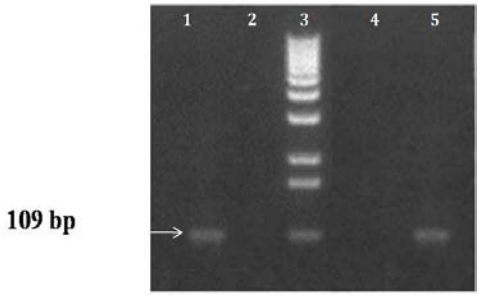

Fig. 1: Figure showing successful amplification of 109 base pair fragment of *16s r RNA* gene of *H. pylori* from the saliva samples of asymptomatic subjects

Table 1: Table depicting the various demographic factors and *Helicobacter pylori* infection in saliva samples of asymptomatic subjects

Variables	No. of subjects	HP positive	HP negative	p value	Odd ratio	95% CI of OR
Gender						
Male	650	377(58%)	273(48%)	1.381	18.36	1.075-1774
Female	400	200(50%)	200(50%)		Referent	
Age						
18-30	168	50(30%)	118(70%)		Referent	
31-45	472	221(47%)	251(53%)	0.0001	2.078	1.425-3.029
46-60	315	151(48%)	164(52%)	0.0001	2.173	1.460-3.335
61-75	95	48(51%)	47(49%)	0.0001	2.41	1.432-4.057
Consumption of alcohol						
Current	493	153(31%)	340(69%)		**Referent**	
Former	45	19(42.22%)	26(57.78%)	0.1343	1.6240	0.8721-3.024
Never	512	269(52.53%)	243(47.47%)	< 0.0001	2.460	"1.901 to 3.184
Smoking						
Current	547	487(89%)	60(11%)	< 0.0001	16.49	11.87 – 22.89
Former	18	6(35%)	12(65%)	1.0	1.016	0.8721-3.024
Never	485	160(33%)	325(67%)	Referent		
NSAIDs use						
Yes	716	594(82.96%)	122(17.03%)	< 0.0001	1 8.36	13.32 – 25.48
No	334	70(20.95%)	264(79.04%)	Referent		
Marital status						
single	368	136(37%)	232(63%)	0.1333	2.291	0.8442 – 6.217
married	647	226(35%)	421(65%)	0.1388	2.255	0.8387- 6.061
divorced	7	3(38%)	4(62%)	0.3201	3.150	0.5275 – 18.81
widowed	26	5(19.23%)	21(80.77%)	Referent		
Area						
Rural	773	486(62.87%)	287(37.12%)	< 0.0001	3.63	2.716 – 4.871
Urban	277	88(31.76%)	189(68.23%)		Referent	
Drinking water						
Raw	583	414(71%)	169(29%)	< 0.0001	11.72	8.566 – 16.59
Boiled/Filtered	352	60(17%)	292(83%)		Referent	
Eating Habit						
Home	370	114(30.81%)	256(69.18%)		Referent	
outside	680	537(78.97%)	143(21.02%)	< 0.0001	8.433	6.325 – 11.24
Socioeconomic status						
Low	244	187(77%)	57(23%)	< 0.0001	24.70	15.52 – 39.32
Medium	516	149(29%)	367(69%)	< 0.0001	3.057	2.038 – 4.585
High	290	34(12%)	256(88%)	Referent		

Statistical analysis performed by Fischer exact test using SPSS version 17. And $p < 0.05$ was considered significant.

RESULTS

The DNA isolated from all the samples were amplified to get a 480 base pair fragment and further amplified to get a 109 base pair fragment in the subjects who had *H. pylori* infection (figure 1). The prevalence of infection in male and female subjects was found to be equal to (58%) and (50%) respectively. The p value was found to be equal to 0.0127 and hence the prevalence was dependent upon gender. The prevalence in age groups of (18-30), (31-45), (46-60), (61-75) was found to be equal to 50%, 47%, 48% and 51% respectively. The p value was found to be equal to 0.0001 and hence age was strongly associated with the disease prevalence.

The prevalence of infection in the population of asymptomatic subjects with respect to consumption of alcohol was as follows: (30.86%) current, (43.15%) former and (52.25%) never. The infection status in

the people who were former consumers of alcohol was not significant (p = 0.1343) whereas it was found to be significant in the people who had never consumed alcohol (p<0.0001). The prevalence of infection in the population of asymptomatic subjects with respect to smoking of cigarettes was as follows: (88.98%) current, (33.33%) former and (32.96%) never. The infection status in the people who were former smokers was not significant (p = 1.0) whereas it was found to be significant in the people who were current smokers (p < 0.0001). The prevalence of infection in the subject population consuming NSAID and not consuming NSAIDs frequently was found to be equal to (82.96%) and (20.95%) respectively. Frequent consumption of NSAIDs was associated with *H. pylori* infection (p < 0.0001). The prevalence of infection in the population of asymptomatic subjects with respect to marital status was as follows: (19.23%) widowed, (34.97%) married, (36.95%) single and (42.85%) divorced respectively. There was no association between the marital status and the prevalence of infection as p > 0.05 in all the groups. The prevalence of infection in the subject who belong to rural and urban area was found to be equal to (62.87%) and (31.76%) respectively. There was significant association between rural residence and *H. pylori* infection (p<0.0001). The prevalence of infection in the subjects who used boiled/filtered water, both filtered and municipal and only municipal, for drinking was found to be equal to (17.04%), (51.30%) and (71.01%) respectively. It was found that *H. pylori* infection had a strong association with consumption of raw water which was not boiled or filtered (p < 0.0001). The prevalence of infection in the subject who eat outdoor and home cooked food was found to be equal to (30.81%) and (78.97%) respectively. It was found that eating outdoors was closely associated with *H. pylori* infection (p < 0.001). The prevalence of infection in the *subject* who belong to high, medium or low socioeconomic status was found to be equal to (11.72%), (28.87%) and (76.63%) respectively. The prevalence of *H. pylori* was significant in the subjects belonging to lower and middle socioeconomic class (p < 0.0001).

The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects with respect to gender was (1.381, 1.075-1.774) in males when females subjects were taken as referent. The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects with respect to age was as follows: (2.078, 1.425 - 3.079) 31-45 years, (2.173, 1.460 - 3.235) 46-60 years and (2.41, 1.432 - 4.057) 61-75 years when subjects of age group 18-30 years were taken as referent. The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects with respect to consumption of alcohol was as follows: (1.624, 0.8721-3.024) former and (2.460, 1.901-3.184) never when current consumers of alcohol was taken as referent. The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects with respect to smoking of cigarettes was as follows: (1.016, 0.3742-2.756) former and (16.49, 11.87-22.89) current when subjects who had never smoked were taken as referent. The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects who frequently consumed NSAIDs was (18.36, 13.32-25.48) when subjects who do not consume NSAIDs frequently were taken as referent. The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects with respect to marital status was as follows: (2.255, 0.8387 - 6.061) married, (2.291, 0.8442 - 6.217) single and (3.150, 0.5275 - 18.81) divorced years when widowed subjects were taken as referent. The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects who reside in rural area was (3.637, 2.716-4.871) when subjects residing in urban area were taken as referent. The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects with respect to treatment of drinking water was as follows: (1.016, 0.3742-2.756) either filtered and boiled, (16.49, 11.87-22.89) municipal when subjects who consumed water which was boiled or filtered were taken as referent. The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects who ate outside was (3.637, 2.716-4.871) when subjects eating at home were taken as referent. The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects with respect to socioeconomic status was as follows: (1.624, 0.8721-3.024) medium and (2.460, 1.901-3.184) low when subjects belonging to high socioeconomic status were taken as referent.

DISCUSSION

H. pylori are spiral or helical in its virulent pathogenic state but are present as a dormant coccoid form in environment [8]. Hence, it is of prime importance to detect the prevalence of *Helicobacter pylori* in the asymptomatic population to predict the possibility of gastrointestinal disorders. The prevalence of *H. pylori* is a domain of research that keeps on changing with an array of variables such as age, gender, consumption of alcohol, smoking, NSAIDs, socioeconomic status urban and rural settings etc. These factors play a major role in the spread of *H. pylori* infection.

This is a pioneer study in Pune region to underscore the relation between prevalence and these variables. *16s r RNA* is a gene which is present in a highly conserved region of *H. pylori*. The results show that smoking, NSAID use, rural residence, consumption of municipal water (not boiled/filtered) and eating outside frequently, enhance the risk of *H. pylori* infection. Alcohol consumption seems to reduce the risk of *H. pylori*. Age, marital status and gender do not influence the prevalence of *H. pylori*.

The observations depict the role of the various demographic factors that are associated with the prevalence of *H. pylori*. The effect of gender on the prevalence of *H. pylori* has been investigated by various authors [9]. However, it was found that there was significant relation between the infection status and gender, which was corroborated in our study. It has been suggested that the infection prevalence increases with the advancing age in the various subjects [10]. Alcohol consumption has been studied in great detail and it has been reported by various authors that the antimicrobial activity of alcohol has an ameliorative effect on the infection status of *H. pylori* [11, 12,13,14,15]. A similar pattern was evident in our study. A strong underlying relation has been found between the smoking of cigarettes and prevalence of infection by previous investigators [12, 13]. Our results exhibit cigarette smoking as a potential risk factor for *H. pylori* infection. NSAID use is closely associated with *H. pylori* infection [15, 18]. Our study showed that frequent NSAID use makes an individual more prone to acquire *H. pylori* infection. It is also evident from our study that prevalence of *H. pylori* in urban and rural population differs significantly. Subjects residing in the rural areas are at a greater risk of acquiring *H. pylori* infection. The source of water has been a major factor in deciding the infection status in various studies [9,19,20]. It was found that subjects consuming municipal water which is not boiled or filtered have an increased chance of acquiring *H. pylori* infection. Socioeconomic status significantly influences the prevalence of *H. pylori* infection [10,24]. In our study it is well elucidated that the subjects of lower socioeconomic status are at a greater risk of acquiring *H. pylori* infection. Food habits also have been reported to influence the prevalence of *H. pylori* [10]. In our study it was observed that the subjects who consumed outside food instead of home cooked food had greater chance and risk of acquiring infection. Marital status has also been investigated to influence the prevalence of the infection [25]. In our study it was found that marital status had no relation with the prevalence of *H. pylori*.

The study also shows that serious measures need to be taken to alleviate this organism from water and food sources. It also suggests that personal hygiene is of optimum importance to inhibit the process of oral oral and oral fecal transmission among subjects. The subjects of lower socioeconomic strata and rural background seem to be at a higher risk of acquiring infection as they are exposed to poor sanitary conditions and consume stagnant unprocessed well water. Our study elucidates the need for proper processing of the potable water by the municipal authorities is indispensable to eliminate the risk of contamination. The proximity of sewage and municipal water pipes may also hold the key to fecal oral transmission of *H.pylori* affecting the asymptomatic subjects as has been elucidated in our study. People need to be aware of stigmas like smoking which clearly demonstrate a correlation of being a promoter of infection susceptibility among asymptomatic subjects. Habitual consumption of NSAIDs seems to have emerged as a regular practice among a large population. It is worth considering that continuous NSAID consumption leads to stoppage of the mucus secretion from the lining of the stomach exposing it to the harsh

milieu of HCl. Hence, it forms a suitable environment to harbor *H. pylori* in the pits where it anchors itself and protects itself from HCl by constructing a structure akin to a defensive umbrella [26].

CONCLUSION

It is concluded that the factors responsible for asymptomatic *H. pylori* infection are smoking, consumption of non filtered and unboiled water, frequent NSAID use, rural residence, low socioeconomic status and frequent consumption of outside food. High socioeconomic class and consumption of alcohol reduce the chance of acquiring *H. pylori*. Gender and marital status does not influence the spread of infection.

Conflict Of Interest

The authors have no conflicts of interest to declare.

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