Rapid diagnosis of *Helicobacter pylori* infection in dyspeptic patients using salivary secretion: a non-invasive approach

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ABSTRACT

Introduction: Current guidelines that recommend *Helicobacter pylori* eradication treatment without endoscopy in selected patients underscore the importance of non-invasive testing. The accuracy of saliva as a non-invasive specimen was compared with that of invasive tests in pretreatment diagnosis of *H. pylori* infection.

Methods: One hundred patients undergoing gastroscopy were grouped into 80 symptomatic and 20 asymptomatic subjects and were investigated for the presence of *H. pylori* in saliva and stomach. Samples tested comprised saliva and gastric biopsies collected from each patient. Exclusion criteria were history of peptic ulcer, bleeding ulcer, cancer or recent use of antibiotics, proton pump inhibitors and non-steroidal anti-inflammatory drugs. Two sets of primers homologous to 534bp fragment of *H. pylori* DNA, which have been shown previously to be highly specific and sensitive, were used for the polymerase chain reaction (PCR) amplification.

Results: 72 (90 percent) of the symptomatic group and 10 asymptomatic subjects were infected with *H. pylori* in the stomach as determined by histology and direct PCR amplification of biopsy DNA obtained from each subject. *H. pylori* DNA was identified in the saliva of 70 (87.5 percent) symptomatic subjects and 12 (60 percent) asymptomatic control subjects.

Conclusion: High rates of detection using saliva as a specimen indicate that saliva of the infected person could serve as a reliable non-invasive alternative to detect the presence of *H. pylori* infection in comparison to the currently available standard diagnostic tests.

Keywords: duodenal ulcer, gastritis, *Helicobacter pylori*, polymerase chain reaction saliva, 16S ribosomal RNA genes

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INTRODUCTION

The gastric pathogen *Helicobacter pylori*, has been a major cause of peptic ulcer disease and is an early risk factor for gastric carcinoma. This gram-negative spiral organism infects the gastric mucosa of over one-half of the world's population and it is the second most common chronic bacterial infection in humans. It has an extremely variable natural history[1]. Although *H. pylori* infection is widespread throughout the world[2], the mode of transmission and other aspects of the epidemiology of *H. pylori* infection still remain unclear. Diagnosing *H. pylori* infection is sometimes difficult.

Conventional methods to diagnose *H. pylori* include culturing the pathogen, microscopical examination, rapid urease test (RUT), and histopathological analysis of the biopsy tissue. All the above-mentioned methods involve gastric biopsy, which requires an endoscopic procedure that in some cases, needs anaesthesia for patients to undergo this test. Secondly, there is a possibility of false-negatives due to sampling error because the culture and histopathology can assess infection only at the biopsy sites[3]. Culture and identifying *H. pylori* in gastric biopsy require experience and dexterity, as identification and culturing are sometimes difficult. Moreover, the erratic distribution of *H. pylori* could also lead to flawed results. Microscopy and RUT can be highly specific if strictly performed, but they are based on biopsy specimens and thus are theoretically prone to sampling error, as in the case of culture[4].

Since invasive methods are expensive, less invasive methods such as serological examination of blood and the urea breath test (UBT) have become more popular[5]. However, positive results by blood serology do not necessarily allow delineation of active *H. pylori* infection. Urea breath tests require expensive specialised equipment and reagents[6], and sometimes become apparently positive in culture negative patients[4,16]. The most commonly used gold standard for *H. pylori* diagnosis, e.g. culture, RUT, histopathology, depends on the gastric biopsy collection. Based on the difficulty of culturing *H. pylori* from sites other

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than the gastric mucosa[7] and the need for non-invasive diagnostic methods, interest has grown in the use of molecular techniques for the detection of this species.

Molecular methods like polymerase chain reaction (PCR) have the potential to accurately determine both the presence of infection and the genotype of bacteria, and have marked sensitivity and specificity[18]. These techniques have been used successfully to detect *H. pylori* DNA in gastric tissues by amplifying genes such as the adhesin genes[9], the urease gene[10] and the 16S rRNA gene[11]. The 16S rRNA gene of *H. pylori* is a highly specific target for amplification and has been used previously to help reclassify the organism. The 16S rRNA is one of the specific targets to confirm *H. pylori* infection, and positive amplification of *H. pylori* specific DNA may be considered as a direct evidence of the presence of the pathogen[4,19-21]. The present study was therefore carried out to standardise a feasible non-invasive method for the rapid diagnosis of *H. pylori* in salivary secretion of infected patients suffering from various gastric maladies using 16S rRNA PCR analysis.

METHODS

The patient population consisted of 100 patients (65 men and 35 women) with a mean age of 48.4 (range 21 to 73) years. The patients were classified at the time of endoscopy into two groups; viz. those having gastric diseases (n=80), and those with no evidence of mucosal ulcer and gastritis (n=20) i.e. normal study. None of the patients had received non-steroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitors (PPI) or antibiotics within the previous two months. Informed consent was taken from the patients who underwent upper gastrointestinal endoscopy at the Department of Gastroenterology, Deccan College of Medical Sciences, Hyderabad, India.

Saliva samples (2-3mL) from 80 symptomatic and 20 non-dyspeptic/non-ulcer patients, along with their gastric biopsies, were collected in a sterile container containing digestion buffer (100mM NaCl, 10mM Tris-HCl (pH 8.0) 0.5%SDS) prior to endoscopy. Three gastric biopsies were collected: one in urea solution for the rapid urease test (RUT), one in normal saline for testing by PCR assay, and one in 10% buffered formalin for histological examination by modified Giemsa stain for the presence of *H. pylori*.

Genomic DNA was isolated from all samples by the cetyl trimethyl ammonium bromide (C-TAB) method according to the standard protocol[12]. Briefly, the frozen gastric biopsy and saliva specimens

were suspended in 250µL of digestion buffer II [0.1M NaCl, 0.01M Tris-HCl (pH 8.0), 0.25M EDTA (pH 8.0), 1% SDS] containing 100µg/mL of proteinase k (Bangalore Genei Ltd, Bangalore, India). To this, 250µL of digestion buffer I [0.1M NaCl, 0.01M Tris-HCl (pH 8.0), 0.25M EDTA (pH 8.0)] was added and incubated at 56ºC overnight. DNA was extracted with an equal volume of phenol-chloroform and precipitated with 0.6 volume isopropanol. The DNA pellets were washed thrice with 80%, 75% and 70% ethanol, respectively, and finally resuspended in 50µL-100µL of TE buffer. Extensive care was taken to avoid contamination during all steps of collecting and preparing the samples.

Two 20-base oligonucleotide primers designated 16S rRNA-F (5'-TAAGAGATCAGCCTATGTCC-3') and 16S rRNA-R (5'-TCCCACGCTTTAAGCGCAAT-3') as reported earlier were selected and synthesised at Bioserve Biotechonolgies (India) Pte Ltd, Hyderabad, India. The amplified product of these two primers with DNAs prepared from the clinical isolates and from the type strain of *H. pylori* (ATCC 26695) was a 534 bp fragment. The specificity of the PCR assay had been previously tested against 10 bacterial strains including three *Helicobacter* species (*H. helmanii, H. mustelae, H. hepaticus*).

PCR amplification was performed as an in-house protocol[13]. Briefly, the template DNA [2µL] was added to 18µL of the reaction mixture containing 1X PCR buffer [50mM KCl, 10mM Tris-HCl (pH 8.3), 1.5% (vol/vol) Triton X-100], 1.5mM $MgCl_2$, 200uM concentrations of each dNTPs, 10pMol of each primer, & 1U of Taq polymerase (Invitrogen Life Technologies, Germany). PCR amplification was performed as previously described[13], which included initial denaturation at 96ºC for 5 minutes, 40 cycles with 1 cycle consisting of 94ºC for 1 minute, 56ºC for 1 second, 72ºC for 2 minutes. The final cycle included a 6-minute extension step to ensure full extension of the PCR products. Amplification was performed in a thermocycler (M J Research Inc, Watertown, USA). DNA of the ATCC 26695 type strain was used as a positive control in each batch of PCR assays while negative control consisted of all the reagents of the master mix except the template DNA.

The PCR-amplified products were analysed by agarose gel electrophoresis. 10µL of each amplified product was added to 3µL of loading buffer (20 mL of glycerol 50%, 25 mg of bromophenol blue, 3 drops of 1N NaOH) and subjected to electrophoresis in a 2% agarose gel. The gel was stained with ethidium bromide and examined under UV transilluminator for the presence of the amplified DNA. Samples were
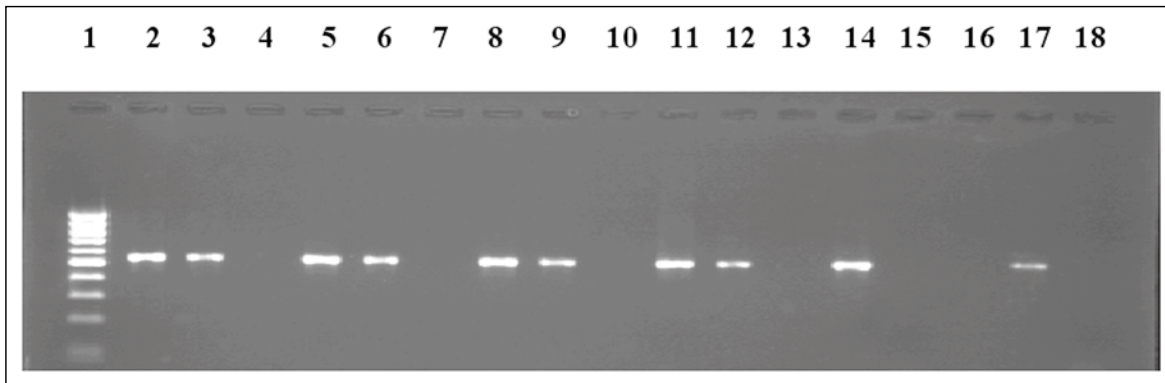
Fig I Gel image shows I6S rRNA amplification of *H. pylori* DNA isolated from the patients. Product size: 534 bp (Lane I represents I00bp molecular weight marker, Lanes 2&3, 5&6, 8&9, I1&12, I4&15 represent I6S rRNA amplification of *H. pylori* DNA isolated from biopsy and saliva of patients, Lane I7 represents positive control (ATCC 26695), Lane I8 represents negative control).

scored as positive when a band of 534 bp could be detected in agarose gel (Fig. 1).

RESULTS

H. pylori was detected in biopsy samples of 72 (90%) of 80 symptomatic patients screened with proven gastric infection by histology. Out of the 20 non-dyspeptic/non-ulcer subjects who were taken as controls, the rate of detection was 50% (10/20). This organism was also detected in saliva samples of 70 (87.5%) of the 80 patients who had active gastric disease proven by histology and biopsy DNA, whereas saliva samples from 12 (60%) subjects in the control group of 20 were found to be positive by PCR. Among these controls, histology could detect *H. pylori* in only nine of the 20 patients screened, and gastric biopsy of ten of the 20 patients indicated active infection. A total of 66 (82%) of the 80 symptomatic patients and eight (40%) of the 20 control group were found to have *H. pylori* infection as confirmed by RUT. Histological examination by the modified Giemsa of the biopsy section showed the presence of *H. pylori* in 70 (87.5%) of the total symptomatic subjects and nine (45%) of the control group, respectively.

DISCUSSION

This study has shown that in more than 87% of cases, *H. pylori* was diagnosed with the help of saliva samples which was comparable with the results obtained from biopsy DNA and histopathological analysis of the gastric tissues of the infected subjects. Further, the presence of *H. pylori* was also diagnosed, using saliva, in asymptomatic subjects who were later found to possess active *H. pylori* infection as confirmed from their gastric biopsy and histopathology obtained after endoscopy.

Currently, there are a number of both invasive and non-invasive diagnostic tests available for the
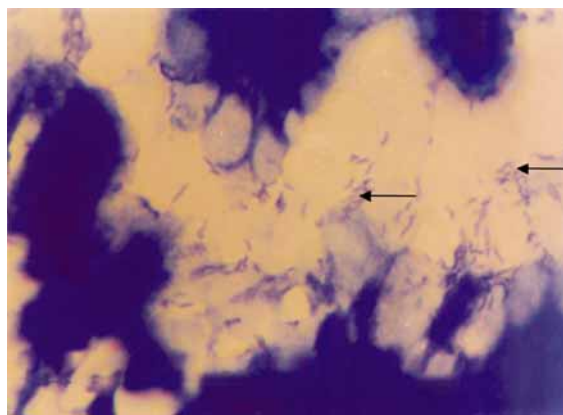

Fig 2 Antral gastric biopsy shows tufts of spiral shaped *H. pylori* (modified Giemsa method).

diagnosis of *H. pylori* infection which have their own sensitivity and specificity, but each has its limitation in clinical applications. Urease-based biopsy tests require endoscopy and are not reliable in cases where patients use proton pump inhibitors. Histological examination follows endoscopy and its accuracy is dependent on the stain selected and on the pathologist's skill. Serology is inexpensive but is not reliable in determining the presence of active infection, which is important for clinical interpretation and diagnosis. Our successful amplification and specific detection of *H. pylori* DNA directly from saliva samples in the majority of infected subjects indicates that this approach is feasible and demonstrates that it has true potential in aiding the diagnosis and management of patients with active *H. pylori* infection.

Earlier attempts made in the past[8] to project saliva as a better specimen for diagnosing *H. pylori* did not generate any comprehensible data, possibly due to low detection rates of *H. pylori* in the salivary secretion and low detection power of the method used. Attempts to isolate *H. pylori* in culture were however not successful from the saliva specimens. The reason could be the environment present in

the mouth effecting the endurance of these organisms due to increased oxygen tension prevalent in these areas. Another reason could be the high contamination load which suppresses the growth of *H. pylori*.

In a previous report from Sweden[14], *H. pylori* could not be cultured from saliva or dental plaque from any of the 52 patients who had culture-positive gastric biopsies. In another report, attempts made to detect *H. pylori* by PCR from saliva and dental plaques showed low rates of detection[11,15,16]. However, a study by Weiss et al[17] on comparative study of the sensitivity, specificity, and predictive value of PCR of formalin-fixed biopsies showed that the 16S rRNA gene of *H. pylori*, has a high accuracy in demonstrating the presence of *H. pylori* in gastric biopsy specimens.

In our study, *H. pylori* DNA was identified by PCR in the saliva of 87.5% of patients, with proven gastric infection confirmed by histological stain of gastric biopsies (Fig. 2) and by DNA isolated from gastric biopsies. In 20 non-dyspeptic/non-ulcer patients (controls), we observed 60% positives for *H. pylori* in the saliva among which 50% were shown to have an active infection as indicated by the biopsy DNA PCR whereas histology and RUT could not confirm these findings.

H. pylori DNA was not identified in the saliva of two (10%) of the non-dyspeptic/non-ulcer patients who had proven *H. pylori* infection as evident from the 16S rRNA amplification of the DNA isolated from their gastric tissues and histopathological findings. The reason for this failure to identify *H. pylori* DNA in the saliva of the two samples is unknown. However, *H. pylori* was also identified by PCR assay in saliva specimens from two control patients whose gastric biopsy DNA were negative by PCR, and histology did not give a clear picture of the presence of bacilli. The relationship between gastric symptoms and *H. pylori* DNA in saliva, however, is unclear. It could be possible that the oral cavity is the initial site of infection. *H. pylori* may persist in low numbers in the oral cavity of these subjects for a long time without colonising the stomach. In a previous study[16]. ample evidence of the presence of *H. pylori* in the oral cavity was put forth. These observations and our findings suggest that the oral cavity could be a reservoir of *H. pylori* infection and oral secretion may be an important means of transmission of *H. pylori*.

The results of our study indicate that *H. pylori* DNA exists in considerably higher amount in the oral cavity and that oral secretion may be an important route of transmission in developing countries. The high rate of isolation of *H. pylori* from the saliva in

the present study indicates that besides being a vehicle of transmission, it may be the prominent source of re-infection or recrudescence. Thus, by determining the type of strain prevalent in the oral cavity or saliva, it could be easy to diagnose the strain colonising the gastric mucosa as reported by Li et al[8] who demonstrated that same strain was present in both niches. Lastly, the economic feasibility of the assay needs to be evaluated in different countries and different settings. In India, where this study was conducted, due to the indigenous synthesis of oligonucleotides by many Indian companies, the cost of such an assay is comparable to other conventional methods such as RUT and microscopy/pathology.

In conclusion, the results of this study indicate that analysis of saliva may potentially be reliable, and could serve as an effective and valuable non-invasive specimen to diagnose and monitor the efficacy of eradication therapy in comparison to the presently available invasive and non-invasive diagnostic tests. This method could be explored to analyse the spread of *H. pylori* which does not necessitate endoscopy to be performed. In addition, this approach may provide a major fillip for further research to assess the age at which this infection is acquired in infants where it becomes almost difficult to perform the other invasive as well as non-invasive tests.

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