



## International Journal of Research in Pharmacy and Life Sciences

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### Research Article

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## Identification of the virulence factor Cag A gene of *Helicobacter pylori* isolated from patients samples

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### ABSTRACT

*Helicobacter pylori* is the human pathogen responsible for the development of gastritis, 15% of the patients may further progress to more severe conditions, peptic ulcer disease and gastric cancer. In fact, depending on the socioeconomic status of the country, the prevalence of infection varies from 40 to over 80% of the population, with higher rates for developing countries. Bacterial virulence factors are essential players in modulating the immune response involved in the initiation of carcinogenesis in the stomach. *Helicobacter pylori* is the most important carcinogen for gastric adenocarcinoma in host genetic factors contribute to the regulation of the inflammatory response and to the aggravation of mucosal damage. A total of 25 samples were subjected to detect "Cag A" gene by PCR analysis. Genomic DNA was isolated from all collected saliva samples by the CTAB. PCR was carried out with specific primers 20 base oligonucleotide primers designated 16S rRNA-F (5'-TAA GAG ATC AGC CTA TGT CC-3') and R (5'-TCC CAC GCT TTA AGC GCA AT-3'). PCR amplification was performed in thermo cycler to ensure full amplification at 40 cycles and PCR products were analyzed by 2% agarose gel electrophoresis. Out of 25 samples, 12 samples were infected with *H.pylori* as detected by Cag A gene as amplified 400bp fragment of *H.pylori*. Identification of Cag A in patients can be considered as a direct evidence of the presence of pathogen. PCR has been used to detect *H.pylori* "Cag A" gene in saliva samples of Indian patients.

**Keywords:** Cag A gene, Genomic DNA, *Helicobacter pylori*, Patient sample, PCR analysis

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## 1. Introduction

The discovery of *Helicobacter pylori* has already changed the natural history of peptic ulcer disease, with most patients being cured at their first presentation. *Helicobacter pylori* (*H. pylori*), the human stomach pathogen, lives on the inner surface of the stomach and causes chronic gastritis, peptic ulcer, and gastric cancer. Plasma membrane repair response is a matter of life and death for human cells against physical and biological damage. *H. pylori* causes plasma membrane disruption injury, and that not only a membrane repair response but also a cell proliferation response are thereby activated. *Helicobacter pylori*'s unique location, persistence, and evasion of the immune system offer important insights into the pathophysiology of the gut. Peptic ulcer disease is a deep gastrointestinal erosion disorder that involves the entire mucosal thickness and can even penetrate the mucosa. In terms of environmental factors, salt intake and smoking contribute to the development of lesions. Various therapeutic schemes are proposed to eradicate *H. pylori* infection, which could potentially prevent gastric cancer, offering the greatest benefit if performed before premalignant changes of the gastric mucosa have occurred.

Earlier analyses of a very few selected gene loci of the gastric pathogen *Helicobacter pylori* indicated that different genotypes predominate in different human populations. The gastric pathogen, *H. pylori*, has been a major cause of peptic ulcer disease and is an early risk factor for gastric carcinoma. It is a known gastric pathogen that infects more than half of the world population [1]. Clinical isolates of *H. pylori* obtained from different individuals and ethnic groups in the world exhibit substantial genomic diversity than other bacterial species [2]. The *H. pylori* infection is very common in India where millions of adults and children are at the risk of developing gastric inflammation, ulcers and carcinoma [3]. To our knowledge, there has been no (or very little) effort made to date to analyze the genetic characteristics of the underlying strains representing Southern India, although studies related to genotypic analyses of isolates from Eastern part of India.

*H. pylori* strains expressing the CagA protein (cytotoxin associated gene A) seem to be more aggressive, inducing either a more severe gastritis or peptic ulcerations, and have been associated

with the development of gastric adenocarcinoma [4]. The finding of anti-CagA antibodies in almost all cases of MALT lymphomas with a significantly higher rate than in active gastritis has led to the hypothesis that CagA1 *H. pylori* strains can be associated with the development of gastric MALT lymphoma [5]. Another study found a significantly higher frequency of CagA1 strain infections in high-grade gastric lymphoma than in low-grade lymphomas or gastritis, suggesting a possible role in histological transformation [6]. However, additional studies were not

able to find any correlation. Hence, the pathogenetic role of the CagA protein remains uncertain, as well as the one of several other *H. pylori* or host proteins that have been suggested as possibly implicated in the MALT lymphoma pathogenesis.

All patients with *H. pylori* infection have histological gastritis, which corresponds to classical chronic gastritis and is characterized by the infiltration of neutrophils and other inflammatory cells. However, most patients are asymptomatic for life, while only some will come to develop a digestive disease [7]. Furthermore, when the relationship between *H. pylori* infection and chronic gastritis was established, investigators began to take interest in the causal role of the bacterium in gastric cancer [8]. It is known that gastric cancer involves the interaction of three major factors: the agent (in the great part of the cases, *H. pylori*) and its pathogenicity, the characteristics of the host, and the external environment [9].

Environmental factors, particularly diet and smoking, play an important role in the pathogenesis of gastric cancer. Diet rich in complex carbohydrates, salt, pickled or smoked foods, dried fish, and cooking oil has been linked with an increased risk, while diet rich in fresh fruits and vegetables has been associated with a low risk of gastric cancer [10]. Smoking also represents an important factor in gastric cancer development. A large study that included smoking men demonstrated an increased risk for the development of differentiated-type distal gastric cancer. The present work focused to identify the presence of CagA as virulence factor in *H. pylori* infected Indian patients and its severity caused plasma membrane disruption injury and also cell proliferation responses were activated against physical and biological damage.

## 2. Materials and Methods

### Bacterial Strains and Culture Conditions

The *H. pylori* (ATCC 43504) strain was obtained from the Post Graduate Institute of Medical Education and Research, Kolkata, India, which were isolated from antral mucosal biopsy specimens of patients with chronic gastritis or duodenal ulcers, and kept as reference in American Type Culture Collection strain (ATCC 43504) were used for this study.

### Collection of Saliva Samples:

The patient population consisted of 25 patients (15 Men, 10 Women) with a mean age of 25 (range 18 to 42) year. The saliva samples (2 ml) from 25 symptomatic ulcer patients were collected in a sterile container, containing digestion buffer. The samples were collected from Government Primary Health Care Center (Villur). The Digestion buffer contains 100mM of Sodium Chloride, 10mM Tris HCL And 0.5% SDS. 25 independent isolates of *H. pylori* were cultured from gastric biopsies obtained after informed

consents from patients of both the sexes with different family backgrounds who have undergone upper gastrointestinal endoscopy and were diagnosed for gastritis and duodenal ulcers. The collected saliva samples were stored in dry ice condition/- 20°C until the further experiment.

*H. pylori* was grown on Columbia blood agar base (BD Difco) containing 5% sheep blood and incubated for 2–3 days in microaerophilic conditions (5% O<sub>2</sub>, 10% CO<sub>2</sub>, 85% N<sub>2</sub>) at 37 °C. The strains were identified on the basis of colony appearance, Gram staining, and positive reactions in biochemical tests (catalase, oxidase, Urea breath test. Stock cultures were maintained until use at –70°C [11].

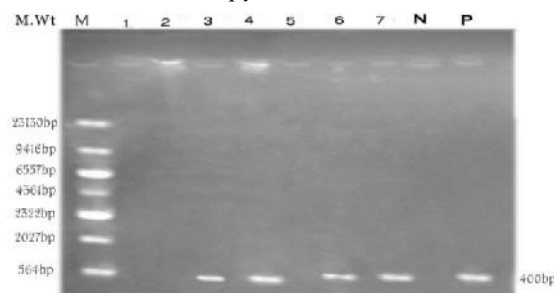
*H. pylori* colonies were identified based on morphology, Gram staining, a positive urease test and subsequent gene-specific PCR tests for the presence of 16S ribosomal RNA gene fragments. Genomic DNA was prepared by the cetyltrimethyl-ammonium bromide (CTAB) method from all the isolates and genomic DNA of *H. pylori* reference strains ATCC 43504 were a gift from Institute of Medical Education and Research, Kolkata, India.

Genomic DNA of saliva samples were checked by 1.2% agarose gel Electrophoresis. PCR was carried out in 50µl volumes containing 10mM Tris HCL (pH 8.3), 50mM KCL, 1.5mM MgCl<sub>2</sub>, 200mM of dNTPs, 50ng of each primers, 2.5 units of Taq polymerase and 5µl DNA template extracted from saliva. PCR was evaluated by using DNA extracted from cultured strains, isolated from the respective patients, as described earlier. Nucleotide sequences of specific primers used are for ‘CagA’ gene was 16s rRNA- F (5’-TAA GAG ATC AGC CTA TGT CC-3’) and R (5’-TCC CAC GCT TTA AGC GCA AT-3’) used to amplification reaction. The amplification reaction normally carried out with a programme consisting of an initial denaturation step at 94°C for 4 minutes, annealing at 59°C for 1 minute and an extension step for 1 minute at 72°C. At the 40<sup>th</sup> cycle included 6 minutes to ensure full extension of the PCR products. Amplification was performed in a minicycler (M J Research Inc). DNA of the *H. pylori* was used as a positive ‘Cag A’ control in each batch of PCR assays. The negative control consisted of all the reagents except the template DNA. 16S rDNA amplification by PCR from template genomic DNA confirmation of amplicon size by agarose gel electrophoresis. The standard *H. pylori* DNA was used as a positive ‘Cag A’ control.

### 3. Results and Discussion

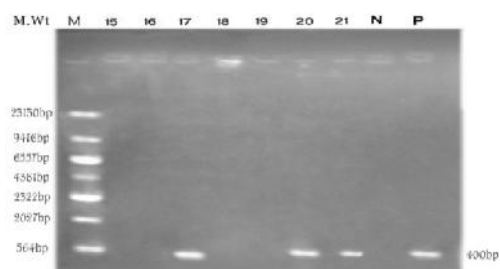
Amplification of CagA gene fragment obtained by using PCR of *H. pylori* infected patients saliva samples DNA as template that yielded the 400 bp amplicon was expected of the *cag A* gene fragment in PCR reaction with specific primers for ‘CagA’ gene was 16S rRNA- F (5’-TAA GAG ATC AGC CTA TGT CC-3’) and R (5’-TCC CAC GCT TTA AGC GCA AT-3’). Positive PCR was obtained with samples from 12 of 25 patients that had scored positive in PCR amplicon. Moreover the method also provided International Journal of Research in Pharmacy and Life Sciences

preliminary *H. pylori* detection directly from infected patients isolated DNA as template for positive amplification of PCR products obtained as results from collected saliva samples of infected patients. Among the 25 individual patients, who gave positive results for the presence of CagA gene in *H. pylori* infection and reference with positive strain showed identical amplified fragment by PCR from DNA extracted from cultured pooled bacterial strains. The other 12 patients were resulted possibly due to Cag A as virulence factor in *H. pylori* strains infection of the host.

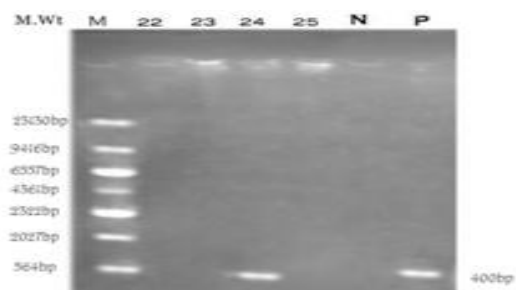


**Figure 1:** Agarose gel showed the amplified product of the genomic DNA.

**Legend:** Agarose gel (2%) showed Cag A gene of the *H. pylori* infection. Lane M: Standard marker (DNA digested with *Hind III*) indicates seven different intense bands of various sizes. Lane 1-7: *H. pylori* strains isolated from different Indian patients samples PCR product. Lane N: Negative control. Lane P: Positive control. Lane: 3, 4, 6, 7 & Lane P Amplification of 400bp fragment of CagA gene as PCR product.



**Figure 3:** Agarose gel shows Cag A gene of the *H. pylori* infection



**Figure 4:** Agarose gel shows Cag A gene of the *H. pylori* infection

**Legend:** 2% Agarose gel showed the amplified product of the genomic DNA.

**Lane M:** Standard marker. ( DNA digest with *Hind III*) indicates seven different intense bands of various sizes.

**Lane 8-14:** *H.pylori* strains isolated from different Indian patients samples PCR product. Lane N: Negative control.

**Lane P:** Positive control. Lane: 8, 10, 11, 14 & Lane P Amplification of 400bp fragment of CagA gene as PCR product.

**Legend:** 2% Agarose gel showed the amplified product of the genomic DNA.

**Lane M:** Standard marker. ( DNA digest with *Hind III*) indicates seven different intense bands of various sizes.

**Lane 15-21:** *H.pylori* strains isolated from different Indian patients samples PCR product. Lane N: Negative control.

**Lane P:** Positive control. Lane: 17, 20, 21 & Lane P Amplification of 400bp fragment of CagA gene as PCR product.

**Legend:** 2% Agarose gel showed the amplified product of the genomic DNA. Lane M: Standard marker. ( DNA digest with *Hind III*) indicates seven different intense bands of various sizes. Lane 22-25: *H.pylori* strains isolated from different Indian patients samples PCR product. Lane N: Negative control. Lane P: Positive control. Lane: 24 & Lane P Amplification of 400bp fragment of CagA gene as PCR product. CagA was initially found as a marker for disease, since patients with antibodies against this protein show higher rates of both peptic ulcers and gastric carcinoma. The disease outcome is determined by multiple factors, including both the bacterial genotype and genetic predisposition of the host. *H. pylori* isolates are surprisingly different in both their genome sequences and their virulence. The reason CagA is associated with disease is still not completely understood. In addition, the benefits of these factors for *H. pylori* itself remain enigmatic. The cellular biology of Cag A and its ability to activate signaling mechanisms and to affect structure, differentiation, and behavior of epithelial cells has become a fascinating area of investigation. Currently, it is known that CagA can activate a number of signal transduction pathways that resemble signaling by growth factor receptors. Simultaneously, CagA is involved in binding and perturbing the function of the epithelial junctions, resulting in aberrations in tight junction function, cell polarity, and cellular differentiation.

*H. pylori* is an excellent model system to study bacterial-induced epithelial cell signaling cascades which are of relevance to neoplasia. A key feature of the increased risk of developing gastric cancer is gastric epithelial hyper proliferation and possibly suppression of apoptosis by chronic *H. pylori* infection. CagA is a major bacterial virulence factor involved in host cell modulation. The presence of a phosphorylation motif in the *H. pylori* translocated effector protein CagA affected the grades of gastric inflammation and atrophy in patients. Dietary factors have the potential to modify *H. pylori* induced epithelial responses and impact on clinical outcome. There is a variation in the cagPAI structure and 3' end of the *cagA* gene in *H.pylori* strain isolated from patients in Tamil Nadu, which may be one of the important virulence

determinants for the variation of the clinical manifestations associated with *H.pylori* infection [12].

High rate of isolation of *cagA* positive *H. pylori* strains from peptic ulcer syndrome patients of Tamil Nadu was observed. The PCR developed here could be of great value in clinical microbiology and *H. pylori* population studies, especially for rapidly screening of many samples to detect of *H. pylori* infections to determine *cagA* status and to identify multiple infections host and detect variant alleles *H.pylori* infection. This interpretation was tested and extended by PCR of *H. pylori* isolates from Tamil Nadu, India. In this study we have shown a rapid mean of identification and differentiation of two strains of *H. pylori* bacteria based on their 16S rDNA gene sequences. Since laboratory diagnosis of *H. pylori* by the existing methods has still less specificity and also it is difficult to diagnose re-infection, this method of detection by 16S rDNA could be a useful alternative. There are several potential advantages to 16S rDNA, PCR and sequencing. The technique is extremely sensitive and can detect DNA from a single infectious agent. Results can be obtained rapidly, unlike culture, which takes days to weeks. Therefore this technique is useful in clinical situations in which conventional microbiologic tests are too insensitive and slow or too cumbersome to be used on a large scale such as *H. pylori* detection. The pathogenicity of *H. pylori* depends on the strain of the bacteria, PCR-Sequencing and analysis of the sequence data by BLAST can be a very quick and useful diagnostic method of the pathogen.

Understanding of gastric carcinogenesis has advanced considerably over the past decades, especially with regards to insights into the role of *H. pylori* infection and the progression of chronic gastritis from premalignant stages to gastric cancer. Based on the results presented whether eradication halts progression or can even cause regression of premalignant gastric lesions, it can be concluded that eradication of *H. pylori* infection has the potential to reduce the risk of gastric cancer development. Furthermore, the optimal time to eradicate the bacterium is before the development of preneoplastic lesions such as atrophic gastritis and intestinal metaplasia. Along with new therapeutic combinations, there is also a need to identify subjects most at risk for cancer from their genetic susceptibility and their infection with *H. pylori* genotypes of greater carcinogenic potential. Finally, early diagnosis is essential to ensure the best outcome of treatment, preventing the development and the worsening of the gastric cancer.

#### 4. Conclusion

In the modern world, *H. pylori* infections are responsible for a heavy toll of morbidity and mortality as a consequence of ulcer disease, lymphoma of the mucosa-associated lymphoid tissue (MALT) and, the most dangerous complication of *H. pylori* infection, gastric adenocarcinoma. These selective pressures operate on a microorganism that possesses specific capabilities for diversification by mutation and recombination. The bacteria

need to interact indirectly and directly with the hosts, requiring both fixed bacterial surface molecules to provide adherence and soluble molecules that are either surface-bound or secreted, and act on their respective host receptors. As such, one might assume that adaptation processes could lead to alterations in three groups of bacterial genes: first, genes in systems that affect intra-bacterial mutation, DNA uptake, repair and recombination themselves; second, genes that favor bacteria–bacteria interactions for the purpose of inter-bacterial genetic exchange, including decreasing or increasing the barrier of genetic exchange; and third, genes that influence bacterial properties that modulate host interaction (adherence and immune response) Indeed, *H.pylori* infection is the leading cause of non-ulcer dyspepsia, peptic ulcers and gastric tumors, including low grade mucosa-associated lymphoid tissue-lymphoma and adenocarcinoma. *H. pylori* infects more than half of the world's human population and gives rise to gastroduodenal diseases, such as peptic ulcer in about 10% and gastric adenocarcinoma in 1–2% of infected people. A novel rapid PCR assay was developed for accurate detection of *H.pylori* infection directly from patient's saliva sample which was subjected for isolation of genomic DNA that could be used as template for amplification of CagA gene fragments as PCR product of 400bp amplicon. Identification of presence or absence of *cagA* gene can be detected in a single reaction, directly from gastric patient's samples without need for culture. It has long been hypothesized that *H. pylori* diversity would be enhanced if humans differ in their food habits (and thereby in gastric environments) and in traits that are important to individual strains such as highly specific immune responses and or availability of receptors helpful in adhesion. However, it depends on patterns of transmission in communities that are dictated by lifestyle and personal hygiene. This is especially true in the Indian context where in spite of a great ethnic diversity there is a lot of similarity in food habits, lifestyle and practice of medicine among the communities.

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