Molecular approach and rapid detection of virulence factor in *Helicobacter pylori* infection and its eradication strategies by Plumbagin

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Abstract

*Helicobacter pylori* is one of the most common pathogens affecting humankind, infecting approximately 50% of the world's population. Of those infected, many will develop asymptomatic gastritis, but 10% develop gastric or duodenal ulcers. The CagA gene is a novel marker for pathogenicity and associated with more severe clinical outcomes and PCR could be rapid and reliable technique to confirm the virulence gene in *H. pylori* infection. 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. *Plumbago zeylanica* root extract were screened for their inhibitory effects on the strains *H. pylori* using the agar diffusion method and showed Plumbagin inhibits the *H. pylori* growth. The minimum inhibitory concentration (MIC) of the potent extracts and minimum bactericidal activity was also observed. The plasmid of *H. pylori* was also isolated and it was treated with the extracts showed the shearing pattern of the plasmid showed its effect against the extracts. *H. pylori* are found in the infected host may be efficiently eradicated by plant based drug such as plumbagin had revealed sheared plasmid DNA of the strains of *H. pylori* used in this study was CagA positive could be a virulence factor in *H. pylori* infection

**Key words:** Cag A gene, gastritis, *Helicobacter pylori*, peptic ulcer, Polymerase chain reaction.

**Introduction:**

*Helicobacter pylori* is the human pathogen responsible for the development of gastritis that, in about 15% of the patients may further progress to more severe conditions, peptic ulcer disease and gastric cancer. Despite the ongoing discussion on which *H. pylori* infected patients should be treated, all colonized patients or just those with overt symptoms of disease, the high rates of prevalence of infection worldwide demand the need for good strategies for eradication. In fact, to depending on the socioeconomic status of the country, the prevalence of infection varies from 40 over 80% of the population, with higher rates for developing countries.

In human, when food is swallowed it passes through the esophagus (the tube that connects the throat to the stomach). It then enters the larger upper part of the stomach. A strong acid secreted from the oxyntic cells of the stomach helps to break down the food. The narrower, lower part of the stomach is known as antrum. The antrum contracts frequently and vigorously, grinding up the food and squirting it in to the small intestine [1]. The duodenum is the first part of the small intestine, just beyond the stomach, which including the antrum is covered by a layer of mucous membrane that protects it from the strong stomach acid.

*Helicobacter pylori* infections are very common worldwide and cause chronic inflammation in the stomach (gastritis), which may progress to peptic ulcer disease and stomach cancer. A peptic ulcer is a sore on the lining of the stomach or duodenum, the beginning of the small intestine. Less commonly, a peptic ulcer may develop just above the stomach in the esophagus, the tube that connects the mouth to the stomach. Peptic ulcers are not caused by stress or eating spicy food, but both can make ulcer symptoms worse. Smoking and drinking alcohol also can worsen ulcers and prevent healing [2]. The bacterium causes peptic ulcers by damaging the mucous coating that protects the stomach and duodenum. Damage to the mucous coating allows powerful stomach acid to get through to the sensitive lining beneath. Together, the stomach acid and *H. pylori* irritate the lining of the stomach or duodenum and cause an ulcer. *H. pylori* is the most common proven risk for gastric cancer and eradication is recommended to reduce the lifetime risk of gastric cancer in patients with atrophic gastritis, in patients after gastric cancer resection and in patients who are first degree relatives of patients with gastric cancer

Other research is exploring how infection spreads from an infected person to an uninfected person. Studies suggest that having contact with the stool or vomit of an infected person can spread *H. pylori* infection [3] and *H. pylori* has been found in the saliva of some infected people, which means infection could be spread through
direct contact with saliva [4]. *Helicobacter pylori (H. pylori)* is a gram-negative, microaerobic rod that lives in the gastric mucous layer, on the surface epithelial cells. *H. pylori* infection causes gastric mucosal inflammation, which could not only lead to chronic gastritis and peptic ulcer disease, but also to gastric adenocarcinoma or low-grade mucosa-associated lymphoid tissue (MALT) lymphoma [5].

*H. pylori* eradication has become a widely accepted initial treatment strategy for stage I gastric marginal zone B cell lymphoma of MALT. Then, it was shown that intentional infection of a volunteer led to gastritis [6]. Classical bacteriology demonstrated that *H. pylori* is a Gram-negative, flagellated organism that produces a number of enzymes, including catalase and urease, which help neutralize host responses and favor colonization [7]. One of the early benefits gained from this basic research was the demonstration that urease is produced by virtually all strains of *H. pylori*. This led to the development of accurate diagnostic tests, including the rapid urease test and the urea breath test. A stool antigen test has also emerged as an informative, noninvasive means by which to diagnose infection via detection of bacterial antigen. In addition, the ability to culture *H. pylori* provided a diagnostic test that allowed assessment of antibiotic susceptibility. In countries with poor sanitation, approximately 90% of the adult population can be infected.

Infected individuals usually carry the infection indefinitely (for life) unless they are treated with medications to eradicate the bacterium. One out of every six patients with *H. pylori* infection may develop ulcers of the duodenum or stomach. *H. pylori* associated with stomach cancer and a rare type of lymphocytic tumor of the stomach called MALT (mucosa-associated lymphoid tissue) lymphoma. *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* also cause plasma membrane disruption injury, and that not only a membrane repair response but also a cell proliferation response are thereby activated. Vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA) have been considered to be major *H. pylori* virulence factors which might therefore provide new insight into potential mechanisms of *H. pylori*-induced gastric carcinogenesis.

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the in vitro antibacterial activity assay [8]. Many reports are available on the antiviral, antibacterial, antifungal, anthelmintic, antollusic, and anti-inflammatory properties of plants [9 and 10]. Medicinal plant has helped in identifying the active principle responsible for such activities and in the developing drugs for the therapeutic use in human beings. Many plants have therefore become sources of important drugs and the pharmaceutical industries have come to consider traditional medicine as a source of bioactive agents that can be used in the preparation of synthetic medicine. In recent times, emphasis is placed on use of natural materials in the control and treatment of various infections as some chemically synthesized drugs have undesirable side effects.

One medicine for reducing the production gastric acid and two classes of antibiotics are used for the combination therapy with three medicines. The first *H. pylori* eradication rate is about only 70% and also the strong side effects by using potent antibiotics are caused. Therefore, by using food which does not have much of anxieties to cause side effect, it is expected that the eradication rate of *H. pylori* will be improved and the strong side effects caused by antibiotics will be reduced while the role of antibiotics will be helped or complemented.

In this study planned to prepare the plumbagin and investigated was also carried out to identify the inhibitor effect of ethanolic extract of *Plumbago zeylanica* on *Helicobacter pylori* in animal model and also present study aimed at evaluating the eradication activity of human *H. pylori* infection for that an attempt was taken to isolate genomic DNA from human saliva samples and subjected to plasmid shearing analyzed in the presence of *Plumbago zeylanica* plant root extracts in order to confirm molecular events to inhibit or eradicate pathogenic *H. pylori* infection.

**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% O₂, 10% CO₂, 85% N₂) 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 cetyl-trimethyl-ammonium bromide (CTAB) method from all the isolates. Genomic DNA of *H. pylori* reference strains ATCC 43504 were a gift from Institute of Medical Education and Research, Kolkata, India.

**Preparation of Genomic DNA by CTAB Method:**

The frozen Saliva samples were taken in centrifuge tube. The samples were spin at −4°C with 10000rpm, until a compact pellet was formed. The pellets were treated with 500µl TE buffer. To this 30µl of 10% SDS and 100µl of proteinase K (concentration is 100µg/ml) was added and incubated for one hour at 37°C 100µl of 5M sodium chloride and 10µl by Cetyl Tri methyl Ammonium Bromide. CTAB solution was added and incubated for 10 minutes at 65°C. Equal volume of chloroform: isoamyl alcohol was added and spin at −4°C with 10000rpm for 5 minutes. This extraction was removed the CTAB and exopolysaccharide complexes. The aqueous layer was transferred in a sterile tube, to add phenol: chloroform: isoamyl alcohol and then spin it at −4°C with 10000rpm for 5 minutes. The supernatant solution was transferred into a fresh centrifuge tube.0.6 volume of isopropanol was added to precipitate the genomic DNA. The DNA pellets were washed with 80%, 75% and 70% of ethanol respectively. Finally it was re suspended in 200µl of TE buffer [12].

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 MgCl2, 200mM of dNTPs, 50ng of each primers, 2.5 units of Taq polymerase and 5µl DNA template extracted from saliva.

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′) 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 40th 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 expect 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.

**H. pylori infection C57BL/6 mice:**

C57BL/6 mice bred in-house were used in all experiments. Experiments were designed to minimize animal suffering and to use the minimum number associated with valid statistical evaluation, according to the guidelines of the animal ethics committee of the institute. Animals were anesthetized by ketamine (12 mg/kg of body weight), followed by cervical dislocation for killing. Animals of both control and experimental groups were kept separately in standard conditions and were fasted for 6 h with free access to water before each inoculation. Groups of mice (12 mice per group) were inoculated with *H. pylori* cultures harvested in PBS twice in a period of 3 days, with about 10⁸ CFU/mouse/inoculation [13]. Mouse groups inoculated with PBS (control group) were kept separately, with free access to water and food. Two weeks after the final inoculation, a group of mice were orally fed with plumbagin (25 mg/kg) once daily for 6 days consecutively, while untreated infected ones received sterile water. All mouse groups were sacrificed 3 weeks post infection, and the gastric tissues were assessed for *H. pylori* colonization and histology.

**Preparation of plant extracts:**

The plant namely *Plumbago zeylanica* (Root) was taken and washed the root section cut into small pieces weighed for about 1gm plus add ethanol to prepare extract with the help of mortar and pestle grind then transfer the extract into eppendroff and keep it for centrifugation at 5000rpm for 15mins at 4°C collect the supernatant and store it for further experimental use. The Agar diffusion method was used to identify the efficiency of the plant extract against pathogen *H. pylori*. (data not given). High performance liquid chromatography (HPLC) was used to purify the plumbagin from crude root extract of *Plumbago zeylanica*. High resolution HPLC was performed using shimadzu LC −10AT up chromatograph provided with isocratic pump and UV visible detector. Column of C18 ODS, Gemini 5 μ, 110A of dimensions 250 x 4.5 mm with mobile phase 70:30:1 (methanol:water : acetic acid), was used at flow rate of 0.5 ml / min. The detection wavelength was 339 nm and injection volume was 20 μl and flow rate 0.9 ml / min , range 0.0100 AUFS
Plasmid DNA isolation:
Plasmid DNA was isolated from the 1.5 ml of \textit{H.pylori} culture the cells were then spun at 6000 rpm for 8 to 10mins. Supernatant was discarded and excess supernatant was drained. The pellet was resuspend in 100ml of ice-cold solution I and vortexed to get a chloroform suspension. The vial was kept on ice bath for 5mins and then shifted to room temperature. To the above suspension 200 ml of solution II was added and mixed by gentle inversion. To the vial 150ml of Solution III was added and mixed and kept for 5mins on ice. Suspension was spin at 6000 to 8000rpm for 5mins at 4°C and supernatant was then transferred to a fresh vial. An equal volume of phenol: chloroform isoamylalcohol (24:25:1) was added to the supernatant. This was vortexed to mix the organic acid and aqueous phase and centrifuged at 10,000rpm for 5mins at 4°C. The aqueous upper layer was transferred in a fresh tube. The plasmid DNA was precipitated from the supernatant by adding (twice the volumes of ethanol at room temperature) and the solution was mixed with vortexing. The plasmid DNA precipitate was collected by centrifugation at 10,000rpm for 5mins at 4°C. The supernatant was completely removed by gentle aspiration. 1ml of 70% Ethanol was added to the pellet and spun at 10,000 rpm for 5mins at 4°C. Supernatant was discarded and the tube was allowed to air drying. After complete drying, the DNA was dissolved in 20ml of TE (pH8.0) buffer containing 20mg/ml of DNase free RNase. The vial was placed at 55°C for 10mins. The plasmid DNA solution can either be stored at -20°C.

Effect of Plumbagin on plasmid DNA:
Shearing effect was also analyzed using plasmid from \textit{H.pylori} as the concentration of plumbagin drug trial experiment was carried out simultaneously to confirm the efficiency of plumbagin against \textit{H.pylori} infection at molecular level. The intensity of plasmid band shows the effect of \textit{P.zeylanica} on plasmid isolated \textit{H.pylori}. Antimicrobial properties of substances are desirable tools in the control of undesirable microorganisms especially in the treatment of infections.

The MIC was considered to be the lowest concentration 100µl, 150µl & 200 µl of the tested sample plumbagin to inhibit growth of \textit{H.pylori} bacteria on the plate, after 24 h. The diameters of the inhibition zones corresponding to the MICs were measured in millimetres with an accuracy of 0.5 mm using a ruler. Each inhibition zone diameter was measured three times (three different plates) and the average was taken. A control \textit{H. pylori} reference strains ATCC 43504 using only inoculation was also carried out.

Histopathology and immunohistochemistry:
Experimental animal stomach tissues were fixed in 10% buffered formalin, embedded in paraffin, and 4 mm sections were prepared, stained with hematoxylin-eosin using standard procedures. Presence of infected organism and gastric lesion has been recorded according to [14].

Results and Discussion:

\textit{Plumbago zeylanica} root ethanolic extract principally contain a yellow naphthaquinone compound namely Plumbagin, which possesses significant activities against wide range of pathogenic bacteria particularly peptic ulcers caused by \textit{H. pylori} are treated with plumbagin drugs that kill the bacteria, reduce stomach acid, and protect the stomach and duodenal lining. Antibiotics are used to kill \textit{H. pylori}. Antibiotic regimens may differ throughout the world because some strains of \textit{H. pylori} have become resistant to certain antibiotics that once destroyed the bacterium is no longer effective. \textit{Helicobacter pylori} is a corkscrew shaped bacteria that resides in the stomach and intestine of more than half of the world’s population, most people have no symptoms. \textit{H.pylori} can cause a number of uncomfortable and painful symptoms including bloating, abdominal pain and vomiting under these conditions patient’s samples such as blood urea, stool, breath test were used for diagnosing the bacteria or an endoscopic exam [Figure 1].

\textit{H. pylori} colonize the stomach of more than half of the world’s population, with infection playing a key role in the pathogenesis of many gastroduodenal diseases. However, not all \textit{H. pylori} strains have the same ability to cause gastric diseases, with host genetic background, environment, diet, hygiene, and cross talk between bacterial gene products and host cells as additional determining factors. Colonization of the gastric mucosa with \textit{H. pylori} results in the development of chronic gastritis in most infected individuals, the clinical outcome of which is dependent on many variables, including \textit{H. pylori} genotype, innate host physiology, host genetic predisposition, and environmental factors. Since \textit{H. pylori} eradication decreases the incidence of gastroduodenal ulcer and prevents its recurrence, CAG and gastric cancer have become the focus of great interest in terms of the feasibility of disease reversal or chemoprevention following eradication of the bacterium. The discovery of \textit{H. pylori} as the causative pathogen of gastric cancer has raised hopes that the malignancy can be prevented through bacterial eradication

These results suggest that \textit{H. pylori} can reach the gastric mucosa capillaries, attach to erythrocytes, and perhaps disseminate throughout the body of humans importantly, the number of bacterial cells in the endothelial lining was drastically lower compared to the foveolar epithelium [Figure1]. This difference in \textit{H. pylori} cell density is consistent with the absence of clinical cases with overt sepsis caused by \textit{H. pylori} infection. Humans can be naturally infected by \textit{H. pylori} which is associated with mucosal inflammation, gastritis and sialylated mucosal
glycosylation pattern. Gastric mucosa of humans was analyzed for spatial localization of *H. pylori* cells. Gentian-stained [Figure 2] revealed the presence of a few *H. pylori* cells tightly associated with erythrocytes within capillaries and post-capillary venules in addition to the previously reported distribution of the bacterial cells in the lumen and foveolar epithelium. Thus, bacterial cells attached to the erythrocyte surfaces were identified by use of probes specific for *H. pylori* 16S RNA, the possibility that *H. pylori* can also invade blood vessels and adhere to erythrocytes in humans was analyzed. In situ hybridization in biopsies of infected human gastric mucosa revealed a similar localization of *H. pylori* bacterial cells. In our study using hematoxylin-eosin, Giemsa and specific *H. pylori* immunostaining no bacteria were found in the control group while in the treatment group a few *H. pylori*-like bacteria were seen [Figure 2]. In order to further evaluate the molecular events, genetics and treatment regimen attempts it is of value to have a model system which is easy to handle. Lymphoma development occurred over a shorter time period in this model than in spontaneous lymphomas are known to appear in aged mice.

Twenty five (25) Patients saliva samples were collected from Primary Healthcare Centre at Vilur in Madurai district. Their samples were used for experimental studies. They were grouped in to two category among the 25 subjects 15 were males (Age range: 18-42) and 10 were females. (Age range: 20-35). 2ml of saliva samples were collected from each individual. The genomic DNA of the patient’s saliva samples were isolated and characterized as *H. pylori* [Figure 3]. The standard *H. pylori* DNA was used as a positive ‘Cag A’ control. The *cagA* gene codes for one of the major *H. pylori* virulence proteins. Bacterial strains that have the *cagA* gene are associated with an ability to cause ulcers. Experimental result revealed, that 12 patient’s DNA containing CagA gene on its genomic DNA sequences, which has been amplified by using right primers, it resulted 400bp amplicon, which can be represent the presence of CagA gene on patient’s saliva sample. In the present studies, out of these 25 patient’s saliva samples 12 samples (3,4,6,7,8,10,11,14,17,20,21&24) consider to have Cag A gene which could be identified. From these 12 suspected samples, samples number 10, 11, 17 & 24 may be expected to have more severity on the basis of the intensity of PCR bands which could be express the severity of the patient’s condition [Figure 4].

Several factors have been proposed as possible virulence determinants. To date, the role of bacterial virulence factors such as vacuolating cytotoxin gene A (*vacA*), cytotoxin association gene A (*cagA*) and lipopolysaccharide (LPS) in the pathogenesis of *H. pylori* infection has been extensively studied. The host’s mucosal immune response, including neutrophil and gastric epithelial activation, has also been the subject of recent investigation. In particular, the *cag* pathogenicity island (PAI), an approximately 400bp region of possibly extraneous origin, is found in about 50–60% of *H. pylori* isolates in Western countries [15] and in more than 90% of such isolates in Japan [16]. Extensive studies of the *cagA* gene, located in the most downstream portion of the *cag* PAI, have demonstrated that the CagA protein is associated with peptic ulcer disease, gastric cancer, and mucosa-associated lymphoid tissue lymphoma. The presence of *cagA* is statistically associated with duodenal ulceration, gastric mucosal atrophy, and gastric cancer. It has also been reported that large sequence differences distinguish the *cagA* gene fragments from Asian strains and other western strains. However, the *cagA* gene does not act alone, because delivery of the CagA protein into epithelial cells appears to be dependent on a secretion system encoded for by the adjacent cluster of genes, the *cag* PAI [17]. After the CagA protein is injected into the host cell cytoplasm, it is tyrosine phosphorylated by host Src kinases and subsequently changes the epithelial morphology [18] CagA in strains from distinct geographic populations appears to be phosphorylated to a different degree, resulting in graded effects on intracellular signaling [19]. *H. pylori* was inoculated in animal for the induction of peptic ulcer and subsequently developed infection was treated by administration of root extract of *Plumbago zeylanica* MICs were determined by the agar dilution method plumbagin inhibited the growth of *H. pylori* infected in experimental animal compared with reference strain ATCC 43504. The antibacterial action of Plumbagin from root extracts of *Plumbago zeylanica* might indicate their potential as antibacterial herbal remedies [20,21]. Research into the effects of local medicinal plants is expected to boost the use of these plants in the therapy against disease caused by the test bacterial species and other microorganisms. It is possible that better therapy for many microbial diseases can be found in the root extracts. The Preliminary results of this investigation indicates that *Plumbago zeylanica* root have high potential of antimicrobial activity against *H. pylori*. Shearing effect was also analyzed using plasmid from *H. pylori* as the concentration of plumbagin [22].

**Differential inhibition of *H. pylori* growth in vitro by plumbagin:**

*Helicobacter pylori* strains were isolated from patients and tested against plumbagin in animal model. The MIC of plumbagin ranged from 100 μg/ml, 150 μg/ml and 200 μg/ml, and the majority of the strains (81%) showed a MIC of either 100 μg/ml (23%) or 150 μg/ml (58%) [Figure 5]. These results clearly confirm that plumbagin acts as a potent growth inhibitor for Indian *H. pylori* strains irrespective of the disease status. However, the roles of polymorphism in target genes and strain-specific differences in the MICs of plumbagin need further investigation against reference strain ATCC 43504. Antimicrobial properties of substances are desirable tools in the control of undesirable microorganisms especially in the treatment of infections. Results obtained showed
that the MIC values for the most sensitive extracts were lower than their MBC values. This suggests that they were bacteriostatic effect at lower concentrations but bactericidal at higher concentrations. Antimicrobial properties of substances are desirable tools in the control of undesirable microorganisms especially in the treatment of infections. The active components were usually interfere with growth and metabolism of microorganisms in a negative manner and has quantified by determining the minimum inhibitory concentration and minimum bactericidal activity. In our study involving hematoxylin-eosin, Giemsa and specific H. pylori immunostaining fewo bacteria were found in the Plumbagin treated group while in the untreated (control) group a few H. pylori-like bacteria were seen. In order to further evaluate the molecular events, genetics and treatment regimen attempts was made evaluate the efficiency of plumbagin to eradicate H.pylori in animal model system incubation occurred over a shorter time period in the experimental design.

*Helicobacter pylori* (*H. pylori*) is a noninvasive, noncore- forming, and spiral-shaped Gram-negative rod bacteria induces infiltration of the gastric mucosa by neutrophils, macrophages, and T and B lymphocytes [23] however, this immune and inflammatory response cannot clear the infection, and leaves the host prone to complications resulting from chronic inflammation [24]. Recent molecular studies reveal that *H. pylori* injected into mice and plumbagin plant component administrated into the cytosol of the gastric host cell via the type IV injection system and regulates the intracellular signal transduction in the host cell [25]. This mechanism provides a novel means of resolving how *H. pylori* survive in the human stomach. During persistent gastrointestinal infections, chronic gastritis may remain asymptomatic or may evolve into more severe diseases, such as peptic ulcer or atrophic gastritis [26]. The use of global gene expression technologies may allow the identification of new therapeutic pathways or molecules that regulate inflammatory process responding to *H. pylori* or reduce oxidative stress to the gastric mucosa. *Helicobacter pylori* is the most important carcinogen for gastric adenocarcinoma. Bacterial virulence factors are essential players in modulating the immune response involved in the initiation of carcinogenesis in the stomach; host genetic factors contribute to the regulation of the inflammatory response and to the aggravation of mucosal damage. In terms of environmental factors, salt intake and smoking contribute to the development of lesions [27]. 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.

**Conclusion:**

Many infectious diseases have been known to treat with herbal remedies throughout the history of mankind. Natural products, either as pure compounds or as standardized plant extracts provides unlimited opportunities for new drug development. The present study was undertaken to elucidate the role of *cagA* genes in Indian isolates for the development of infection also to identify whether differences in this region would be present in *H. pylori* isolates from patients with different *H. pylori*-related diseases in India. 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 ‘CatA’ 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 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. *H. pylori* strains expressing the CagA protein (cytotoxinassociated 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. Therapeutic gastroscopes and colonoscopes provide options for auxiliary water channels or secondary instrument channels, each creating additional steps in the cleaning and reprocessing procedures. Therapeutic gastroscopes may have double therapeutic channels, each needing to be brushed, cleaned and disinfected. They may require additional adapters in order to effectively disinfect the endoscope.

The active plumbagin components usually interfere with *H pylori* growth and metabolism of microorganisms in a eradication manner and is quantified by determining the minimum inhibitory concentration and minimum bactericidal activity. 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 Cag A gene could be obtained rapidly in different culture therefore PCR amplification technique was useful in clinical situations in which conventional microbiologic tests were too insensitive and slow to be used on a large scale specifically in *H* pylori detection. The pathogenicity of *H pylori* depends on the strain of the bacteria virulence factors sequencing and analysis of the sequence data by BLAST can be a very quick and useful diagnostic method of the pathogen.
Medicinal plant extracts approach to the treatment of *Helicobacter pylori* infection also plumbagin treatment trail experiment for *H. pylori* eradication in animal model have yet to be established as no clear evidence mechanism existed in that eradication of the bacterium results in ulcer prevention and gastric inflammation persists even after successful eradication. This has led to increased interest in molecular approaches to the treatment of *H. pylori* infection, including plumbagin may be the active agents in the therapeutic benefits of plumbagin supplemented in *H. pylori* infection. The plasmid was also isolated from *H pylori* and they were treated with ethanol root extracts of *Plumbago zeylanica* shows the shearing pattern of the plasmid thereby confirm its antibacterial effects against the infection. Plant based antimicrobials have enormous therapeutically potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials.

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**References:**

Figure 1: Scanning electron micrograph of *H. pylori*.

**Legends:** *H. pylori* are helix-shaped Gram-negative bacterium, microaerophilic about 3 micrometres long with a diameter of about 0.5 micrometres. *H. pylori* has 4–6 flagella; all gastric and entero hepatic *Helicobacter* species are highly motile due to flagella.
**Legends:**

Genta-stained section of human gastric biopsy. Black spiral- and comma-shaped bacteria are observed in the lumen of the stomach, adherent to gastric epithelial cells, within the mucus globule of the cells. Bacterial cells (arrow) are also present in close contact to an erythrocyte within a capillary located in the supporting connective tissue of lamina propria of the mucosa.

**Figure 2: H. pylori adheres to erythrocytes in capillaries of infected sample**

**Figure 3: Genomic DNA isolated from human saliva**

**Legends:** Genomic DNA isolated from *H. pylori* infected human saliva samples were separated on 1.2% agarose gel. **Lane M:** Standard marker (λ DNA digest with Hind III) indicated ranges of molecular weight size intense bands. **Lane 1-25:** Genomic DNA extracted from patient’s saliva shows one intense band of DNA at 23Kb.
**Legend:** Agarose gel (2%) showed Cag A gene of the *H. pylori* infection

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

Lane 1-25: *H. pylori* strains isolated from different Indian patients samples PCR product.

Lane N: Negative control. Lane P: Positive control.

Lane: 3, 4, 6, 7, 8, 10, 11, 14, 17, 20, 21 & 24 indicated Amplification of 400bp fragment of CagA gene as PCR product.
Figure 5: Shearing effect of Plumbagin plasmid isolated from infective strain

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Legends: Lane 1: Molecular weight marker 1Kb
Lane 2: Control *H. pylori*
Lane 3: *H. pylori* plus 100μl of plumbagin
Lane 4: *H. pylori* plus 150μl of plumbagin
Lane 5: *H. pylori* plus 200μl of plumbagin

Figure 6: Plumbagin showed Minimum inhibitory (MIC) effect on *H. pylori*

Legends: *Plumbago zeylanica* root extracts contains plumbagin was purified by using HPLC then the active component has tested against *H. pylori* inoculated on agar plate. After 3 days MIC were observed and confirmed its inhibitory action anti ulcer mechanism which lead to the development of plant based drug for eradication of *H. pylori* infection.
Legends:

The body and the pyloric parts of stomachs from control mice and mice infected for 3 weeks and treated with plumbagin were sectioned for histological studies. The tissue samples were fixed in 10% formalin and embedded in paraffin. The sections (5 μm) were cut with a microtome, stained with hematoxylin and eosin and Giemsa stain for gastric tissue observed under microscope. Images were captured at original magnifications of ×10 and ×20. Hematoxylin-eosin stain and Giemsa stain for gastric tissue showed presence of few H. pylori in the plumbagin-treated mice and control has showed various number of H. pylori in the mucus as well as in some glands.