Molecular cloning of iceA1 gene of Helicobacter pylori in relation to gastric cancer

SALIMA SALEH MOHAMED ALGAIAT¹, SAPNA SMITH LAL²

Abstract: H. PYLORI are spiral-shaped bacteria that grow in the digestive tract and have a tendency to attack the stomach lining. H. PYLORI infections are usually harmless, but they’re responsible for the majority of ulcers in the stomach and small intestine. H. PYLORI is a common type of bacteria that usually infects the stomach. They may be present in more than half of all people in the world, according to the Mayo Clinic. The “H” in the name is short for HELICOBACTER. “Helico” means spiral. The bacteria are spiral shaped. H. PYLORI normally infect your stomach during childhood. While infections with this strain of bacteria typically don’t cause symptoms, they can lead to diseases in some people, including peptic ulcers, and an inflammatory condition inside your stomach known as gastritis. H. PYLORI are adapted to live in the harsh, acidic environment of the stomach. These bacteria can change the environment around them and reduce its acidity so they can survive. The shape of H. PYLORI allows them to penetrate your stomach lining, where they’re protected by mucus and your body’s immune cells are not able to reach them. The bacteria can interfere with your immune response and ensure that they’re not destroyed. This can lead to stomach problems.

Keywords: H. PYLORI, HELICOBACTER, Helico.

I. INTRODUCTION

Helicobacter pylori is a helix-shaped (classified as a curved rod, not spirochete) Gram negative bacterium, about 3 micrometers long with a diameter of about 0.5 micrometres. It is microaerophilic; that is, it requires oxygen, but at lower concentration than is found in the atmosphere. It contains a hydrogenase which can be used to obtain energy by oxidizing molecular hydrogen (H2) that is produced by intestinal bacteria. It produces oxidase, catalase, and urease. It is capable of forming biofilms and can convert from spiral to a possibly viable but non cultural coccoid form, both likely to favor its survival and be factors in the epidemiology of the bacterium. The coccoid form can adhere to gastric epithelial cells in vitro. Helicobacter pylori possess five major outer membrane protein (OMP) families. The largest family includes known and putative adhesions. The other four families include porins, iron transporters, flagellum-associated proteins and proteins of unknown function. Like other typical Gram-negative bacteria, the outer membrane of Helicobacter pylori consists of phospholipids and lipopolysaccharide (LPS). The O antigen of LPS may be fucosylated and mimic Lewis blood group antigens found on the gastric epithelium. The outer membrane also contains cholesterol glucosides, which are found in few other bacteria. Helicobacter pylori has 4-6 lophotrichous flagella; all gastric and enterohepatic Helicobacter species are highly motile due to flagella. The characteristic sheathed flagellar filaments of Helicobacter are composed of two copolymerized flagellins, FlaA and FlaB. The most striking biochemical characteristic of Helicobacter pylori is the abundant production of urease.

This bacterium colonizes gastric mucosa and elicits both inflammatory and immune lifelong responses, with release of various bacterial and host-dependent cytotoxic substances. Pathological studies and extensive clinical trials, carried out in the past few years, have proved the causative role of H. pylori in the development of chronic gastritis (Peterson et al., 1998) and peptic ulcer disease (Cohen et al., 2000). It seems that this bacterium is also causally related to low-grade B-cell lymphoma of gastric mucosa-associated-lymphoid-tissue [MALT-lymphoma] (Par sonnet et al., 1994). Moreover, H. pylori infection has been established as a risk factor for the development of both diffuse and intestinal types of gastric cancer (Xue et al., 2001). Recent studies suggest an epidemiological association between H. pylori infection and several extra gastro duodenal pathologies, including cardiovascular, skin, rheumatic and liver diseases. Unfortunately, such epidemiological studies are influenced by a wide variety of confounding factors,i.e. socioeconomic status, time of acquisition of the infection, presence of different bacterial strains and previous antibiotic therapy. However, according to many authors, the observed associations might be true and explained by a role of H. Pylori infection in the pathogenesis of certain extra digestive disorders. It is well known that H. pylori colonization of the gastric mucosa stimulates the release of various proinflammatory substances, such cytokines, eicosanoids and proteins of the acute phase (Crabtree, 1998).

Moreover, a cross mimicry between bacterial and host antigens exists in H. pylori infected patients (Realdi et al.,
Therefore, a pathogenic link between *H. pylori* infection and diseases characterized by activation of inflammatory mediators and/or induction of autoimmunity might exist. Chronic inflammation and increased immune response have been observed in a variety of respiratory diseases, including chronic bronchitis and bronchiectasis. Moreover, both chronic obstructive pulmonary disease and pulmonary tuberculosis are more prevalent in peptic ulcer patients than in the general population. Based on these facts, many recent studies have focused on the potential association between *H. pylori* infection and various respiratory disorders (Gasbarrini et al., 1999). Helicobacter pylori is one of the most common bacterial infections worldwide. At least 50% of the world’s population is infected. The prevalence of *H. pylori* infection in a community is related to three factors, the rate of acquisition of infection, i.e. the incidence, the rate of loss of the infection and the prolonged prevalence of the bacterium in the gastro-duodenal mucosa between infection and eradication. Acute *H. pylori* infection invariably passes undetected. Thus, the incidence of infection is determined indirectly from epidemiological studies. The incidence of *H. pylori* infection is estimated to be approximately 0.5% per year in adults of developed countries. This incidence has been decreasing over time. However, the incidence of *H. pylori* infection continues to be high in developing countries.

II. MATERIALS AND METHODS

A. Isolation of genomic DNA from bacteria

Total genomic DNA from the bacteria was isolated by N-Cetyl-N,N,N-trimethyl-ammonium bromide (CTAB) method.

**Composition of Extraction buffer**

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>Buffer composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M TrisHCl</td>
<td>100 mMTrisHCl</td>
</tr>
<tr>
<td>1M EDTA</td>
<td>100 mM EDTA</td>
</tr>
<tr>
<td>4 M NaCl</td>
<td>1.4 M NaCl</td>
</tr>
<tr>
<td></td>
<td>1% CTAB</td>
</tr>
<tr>
<td></td>
<td>Proteinase K - 0.03μg/μl</td>
</tr>
</tbody>
</table>

SDS 20% w/v

Chloroform: isoamyl alcohol (24:1)

Isopropanol

Ethyl alcohol 70% v/v

**Quantification of Isolated DNA**

The quantity of the isolated DNA was checked in UV-VIS spectrophotometer (Vivaspec Biophotometer, Germany). From the stock 1μl DNA was mixed with 49-μl sterile distilled water to get 50 times dilution. The A260/A280 ratio was recorded to check the purity of DNA preparation.

**PCR Amplification**

Reagents and the optimal PCR reaction mixture

<table>
<thead>
<tr>
<th>PCR components</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease free water</td>
<td>10.70</td>
</tr>
<tr>
<td>10X reaction buffer with MgCl₂ (1.5mM)</td>
<td>2.00</td>
</tr>
<tr>
<td>dNTP mix (2,5mM)</td>
<td>2.00</td>
</tr>
<tr>
<td>Primer I (10picomoles/μl)</td>
<td>2.00</td>
</tr>
<tr>
<td>Primer II (10picomoles/μl)</td>
<td>2.00</td>
</tr>
<tr>
<td>Taq DNA polymerase (5U)</td>
<td>0.3</td>
</tr>
<tr>
<td>Template DNA (50ng/μl)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**Total volume**: 20.0 μl

**PCR temperature profile**

- Initial Denaturation: 94°C for 2 min
- Denaturation: 94°C for 50 s
- Annealing: 52.5°C for 30 s
- Extension: 72°C for 1 min
- Final extension: 72°C for 6 min

30 cycles

**Table showing the details of the forward and reverse primers designed towards amplification of the iceA gene of Helicobacter pylori.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5'-3')</th>
<th>GC %</th>
<th>Tm Value</th>
<th>Length</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP</td>
<td>CAT TGT AGG AGC CAA CCT CTI</td>
<td>52.4</td>
<td>59.7°C</td>
<td>21</td>
<td>500 bp</td>
</tr>
<tr>
<td>RP</td>
<td>CTA CAG GTG CCA TGA TTT CC</td>
<td>50.0</td>
<td>62.9°C</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

**50X TAE buffer**

**Chemicals**

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>242g</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>57 mL</td>
</tr>
<tr>
<td>500mM EDTA (pH 8.0)</td>
<td>100mL</td>
</tr>
<tr>
<td>Water</td>
<td>volume up to 1 liter</td>
</tr>
</tbody>
</table>

**Ethidium Bromide**-10 mg/ml (Keep in dark at 4°C)

**Protocol**

- 1g agarose powder was measured and taken in a 250 ml conical flask
- 100 ml TAE Buffer was added to the flask and mixed properly.
- The agarose was melted in a microwave or hot water bath until clear solution was obtained.
- The solution was allowed to cool to about 50-55°C, by swirling the flask.
- 5μl of ethidium bromide solution was added to the melted agarose gel and mixed well.
- The ends of the casting tray were sealed with two layers of tape.
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- The combs were placed in the gel casting tray.
- The melted agarose solution was poured into the casting tray and was allowed to solidify.
- The combs were pulled out carefully and the tapes were removed.
- The gel was placed in the electrophoresis chamber.
- Enough TAE Buffer was added so that there is about 2-3 mm of buffer over the gel.
- 6 µl of 6X Sample Loading Buffer was added to each 25 µl PCR reaction.
- Each sample/ DNA ladder was carefully pipetted into separate wells in the gel.
- The electrode wires were connected to the power supply, making sure the positive (red) and negative (black) were connected correctly.
- The power supply was set to about 100 volts.
- The power supply was turned off after the samples had run sufficiently, the gel was removed using gloves and visualized under U.V. light, photographed and documented using UV trans-illuminator.

DNA extraction from Agarose gel

- About 300mg Agarose gel fragment was excised with scalpel.
- The gel slice was transferred to 1.5ml or 2 ml tube.
- 650µl Gel Solubilizer was added.
- Sample was incubated for 10 min at 50°C to dissolve slice and vortexed.
- 50µl Binding Optimizer was added and vortexed.
- 750µl of sample was transferred to Spin Column A placed in Collection Tube.
- Sample was centrifuged at 10000g for 1 min. Filtrate was discarded and collection tube was reused.
- Any residual solution was loaded and step 7 was repeated.
- 700µl Wash Buffer A was added.
- Sample was centrifuged at 10000g for 1 min. Filtrate was discarded and Collection Tube was reused.
- Steps 9 and 10 were repeated.
- Sample was centrifuged at Max speed for 2 min and Collection Tube was discarded.
- Spin Column A was placed in 1.5ml Elution Tube.
- 30-50µl Elution Buffer was added directly to Spin Column membrane.
- Sample was incubated at room temperature for 1 min.
- Sample was centrifuged at 6000g for 1 min to elute DNA.
- The eluted DNA was stored at 4°C, until further use.

Cloning of the iceA gene

Reagents for Transformation of E. coli

Ampicillin stock solution (50mg/ml)

| Ampicillin | 1g |
| Distilled water | 5ml |

Sterilized in a 0.22µm filter, made into aliquots and stored at -20°C

IPTG (Isopropylthio-β-D-galactoside) solution

20% (w/v) Sterilized in a 0.22µm filter, made into aliquots and stored at -20°C.

X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside)

X-gal 20mg in 1ml of N, N’ dimethyl formamide. Sterilized in a 0.22µm filter, made into aliquots. The solution was stored at -20°C in an amber coloured vial.

Bacterial strain

E. coli strain DH 5-α was used in the cloning work.

Preparation of chemical competent E. coli cells (Sambrook and Russel 1989).

The competent cells for the introduction of recombinant plasmids were prepared using the following protocol. A single colony of E. coli DH-5-α was inoculated to 5ml of Luria Bertani (LB) broth and incubated overnight at 37°C in an orbital shaker with an agitation of 200rpm. From the overnight culture (12-16h), 500 µl were added to 50 ml of LB broth and incubated at 37°C with moderate agitation (200rpm). Optical density was checked at A600 during different intervals until the O. D. reached about 0.375. Then the cultures were transferred to pre-chilled centrifuged tubes and incubated for 10 minutes on ice. The tubes were centrifuged at 3000g for 10 minutes at 4°C. The supernatant was discarded carefully and the pellet was resuspended gently in 10 ml of 0.1M MgCl₂ solution without vortexing and the cells were incubated for 1 h on ice. The tubes were again centrifuged at 3000g for 10 minutes at 4°C. The supernatant was discarded carefully and resuspended the pellet gently in 1ml of 0.1M CaCl₂ solution. The tubes were incubated on ice for 1 hour. From the final preparation, aliquots of 200 µl were prepared in microcentrifuge tubes and kept on ice or used immediately. The remaining competent cells were stored at -80°C by mixing equal volume of 40% glycerol.

Transformation

The ligated product was mixed with 200µl of prepared competent cells and incubated on ice for 30 minutes without disturbing. Heat shock was given to the ligation and competent cell mixture at 42°C for 2 minutes. The tubes were transferred quickly onto ice and incubated for 2-3minutes. To the mix, 1ml of LB broth was added and the tubes were placed in an orbital shaker at 37°C for 1 hour with an agitation of ~200rpm.During the incubation period, 50ml of LB agar was melted and allowed to cool to 40°C. To the 50ml of molten LB agar, 50 µl of Ampicillin (50mg/ml) was added to a final concentration of 50 µg/ml, 200 µl of X- gal, to a final concentration of 80µg/ml and 20 µl of IPTG to a final concentration of 80 µg/ ml. The molten agar was mixed properly without forming air bubbles and poured on to the sterile Petri plates. The plates were allowed to solidify for 10-15 minutes and were incubated at 37°C until plating. After 1 h incubation in orbital shaker, the tubes containing cells were...
centrifuged at 1000rpm for 10 minutes at room temperature and resuspended the pellet in 100 μl of fresh LB broth. From the suspension, 100 μl was spread on LB agar plate using a bent sterile glass rod. The plates were incubated at 37°C overnight.

Screening of positive clones
White colonies containing recombinant plasmids due to the insertional inactivation of the lacZ gene were selected and streaked on a fresh LB plate containing ampicillin and incubated overnight and served as a master plate for each transformant. All colonies from the master plate were subjected to plasmid DNA isolation and restriction analysis to identify the positive recombinants.

III. RESULTS AND DISCUSSION
Genomic DNA isolation and quantification
The Gram negative bacteria were cultured in the Brain heart infusion broth media and genomic DNA was isolated by modified CTAB method. The isolated DNA was electrophoresed in 1% Agarose gel (Fig.4). The quantity and quality of DNA was analyzed by UV visible spectrophotometer and the data was shown in the table 5. A260/280 value showing the purity of the isolated DNA.

Concentration of the isolated genomic DNA

<table>
<thead>
<tr>
<th>DNA Sample</th>
<th>Concentration (ng/μl)</th>
<th>A260/280</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>115.0</td>
<td>1.72</td>
</tr>
<tr>
<td>2</td>
<td>352.0</td>
<td>1.73</td>
</tr>
<tr>
<td>3</td>
<td>318.0</td>
<td>1.84</td>
</tr>
</tbody>
</table>

PCR amplification of the iceA gene
The H. pylori iceA genetic locus was identified because transcription of the iceAI allelic variant of this gene was induced following adherence of the organism to gastric epithelial cells in vitro. This observation suggested to us that iceAI might be a virulence-associated gene, because epithelial cell contact induces the expression of genes associated with virulence in other gastrointestinal pathogens. Species specific primers were designed for the Helicobacter pylori using the sequences of iceA gene available in NCBI GenBank using Primer 3 Software. The predicted primers were validated initially in silico and subsequently in wet lab. The primers could yield an amplicon of the expected size specific to iceA gene. The PCR product was electrophoresed and visualized by 1% agarose gel. The primers were found to produce ~1060 bp amplicon which shown in the figure 2. Epidemiological evidence showing an association of iceAI strains with enhanced gastric inflammation and duodenal ulcer disease.

Fig2. PCR amplification of ice A partial gene by specific primers (M- 1kb ladder, Line 1, 2, 3- ice A gene product, NC- Negative control).

Cloning of PCR product in to T vector
PCR yielded a specific amplicon of 1060-bp in Helicobacter pylori strain (Fig. 2). The fragment was separated in gel and purified the product using gel extraction kit. The purified PCR product was quantified and ligated with cloning vector (Fig. 3) using T4 DNA ligase enzyme. The ligated plasmid was transformed in to E.coli bacterial strain DH5α. The transformation was done by heat shock method and transformed cell was cultured in the Xgal-IPTG-ampicillin-LB Agar plate at 37°C for overnight (Fig.4). The white colonies were picked up from the plates and cultured in Ampicillin containing LB broth.

Fig3. Map of T vector pTZ57R/T (Fermentas, Germany).
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Sequence data
The gene was identified by sequencing of plasmid. An approximately 1060-bp region of the iceA gene was sequenced at Eurofins, Bangalore. The sequence data was shown below. Nucleotide sequence analysis of gene was used to investigate the identity of bacterial iceA gene of Helicobacter pylori. To demonstrate the quality and accuracy of results provided from a public database, we compared sequences to their corresponding GenBank sequences. The sequence had “perfect” match (similarity, 99%) with sequences of their corresponding gene (iceA1) from GenBank as determined by using BLAST (version 2.7).

Confirmation of clone by restriction digestion
The purified plasmid was subjected to restriction digestion using Bam H1 and EcoR 1 (Merck, India). After incubation at 37°C for 4 hours the restricted product was electrophorized on 1% Agarose gel (Fig. 6).

IV. SUMMARY AND CONCLUSIONS
Marshall and Warren’s seminal discovery that a humble bacterium, Helicobacter pylori, causes gastritis, peptic and duodenal ulcers, and in some cases, gastric cancer, merits the 2005 Nobel Prize in Physiology or Medicine for its remarkable impact on public health and for opening up new
avenues of research. Helicobacter pylori infection induces a histologic gastritis, even in asymptomatic individuals with both acute and chronic characteristics. Because Helicobacter pylori is a mucosa-associated organism, it was initially thought that an IgA type anti-Helicobacter antibody response would be essential for protective immunity. Subsequent studies provided compelling evidence that a humoral response was not required for protective immunity against gastric helicobacters. The lack of a protective effect by IgA antibodies may be supported by the observation in humans that the prevalence of Helicobacter pylori infections does not differ between IgA deficient individuals and persons with normal IgA levels. The only modality available for Helicobacter pylori eradication today is by the use of multidrug cocktails that have the disadvantages of compliance and organism resistance. Because eradication regimens have various shortcomings, the only real effective way of dealing with Helicobacter pylori is prevention by way of immunization. In the present study we cloned and characterized the ureA gene from Helicobacter pylori to develop an antigen for the protective immunity against gastric Helicobacters. The iceA2 gene from Helicobacter pylori was amplified using specific primers and cloned in pTZ57R/T and transformed into DH5α cells. The plasmid DNA obtained was then confirmed by restriction digestion and sequence analysis. The sequence was found to be 99% similar to that obtained in GenBank. The sequence of iceA2 gene amplified by the specific primer is closely matching (99%) with a generic primer is closely matching (99%) with a

V. REFERENCES


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