Cloning and Expression of iceA1 Gene of Helicobacter pylori.

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ABSTRACT
Helicobacter pylori, a gram-negative, motile, spiral-shaped bacterium, have been established as the etiologic agent of chronic gastritis and belongs to phylum Proteobacteria. The gene iceA (induced by contact with epithelium) is also proved to be an important factor for the pathogenicity. The iceA1 gene is usually expressed when Helicobacter comes in close contact with the epithelial cells of the gastric lining. Moreover its presence has also been identified in those patients suffering from peptic ulcers. Due to the fact of its involvement in the contact to the epithelial layers, iceA gene has been focused primarily in the disease diagnosis. Characterization and cloning of this gene could be a potential mode of vaccine development towards this gastric cancer. And moreover the antibiotic based drugs are of limited use. Novel methods are being developed for the production of antibodies to specific antigens and thus helping in the process of development of protein based vaccines. iceA gene was isolated and ligated into pTZ57R/T cloning vector. The ligated product was then cloned into DH5α strain and allowed to propagate. The plasmids thus cloned were purified and later expressed for the gene of interest in an expression vector. The proteins specific to the gene of interest was then isolated and purified. This proteins purified can in turn be used for protein based vaccines.

Key words: Helicobacter pylori, iceA gene, Cloning, Restriction digestion.

INTRODUCTION
Helicobacter pylori infection is now accepted as the major cause of chronic gastritis7,17. Several epidemiological studies have shown that H. pylori infection is also linked to severe gastritis-associated diseases3,4 including peptic ulcer disease (PUD) and gastric cancer (GC)20. The infection remains latent in the majority of infected patients, with only approximately 20% of infected individuals developing severe diseases2,3. In addition to host, environmental, and dietary factors, another possible reason for the various outcomes of H. pylori infection relates to differences in the virulence of H. pylori strains21.

Helicobacter pylori is a microaerophilic, gram negative spiral shaped bacterium that colonizes the human stomach3,6. It has been proved of its association with chronic gastritis, peptic ulcers and gastric cancer8. It has also been found to play a vital role in mucosa-associated lymphoid tissue lymphoma3,14. WHO has assigned H. pylori a definite class I carcinogen16. Even though the rate of prevalence of H. pylori is more in the developing countries18,37 only a minute proportion of the population develop severe disease.

Helicobacter pylori lives within the gastric layer as such it is well adapted to grow in an acidic environment7. The bacteria usually make the epithelial layers more susceptible to the acid attack, which in turn leads to severe inflammation11. This can be due to the involvement of several factors in the development of pathogenicity. cagA (cytotoxin-associated gene), vacA (vacuolating cytotoxin gene) are some of the genes which play a major role in the infection. iceA (induced by contact with epithelium) gene is also proved to be an important factor for the pathogenicity12,18. Induced contact with epithelium (iceA) gene has two alleles namely iceA1 and iceA217. These two genes and its association with the pathogenicity are not yet certain. Their role has not yet been identified. The iceA1 gene is usually expressed when Helicobacter comes in close contact with the epithelial cells of the gastric lining. Moreover its presence has also been identified in those patients suffering from peptic ulcers21,22. But the iceA2 was found to be more prevalent in those patients who do not have any ulcers and are infected with Helicobacter23.

Isolation and characterization methods have been employed to diagnose the disease. Many of the molecular approaches have been targeted towards the genes of Helicobacter for accurate and recurrent infections caused by the pathogen16,19. Due to the fact of its involvement in the contact to the epithelial layers, iceA gene has been focused primarily in the disease diagnosis. Characterization and cloning of this gene could be a potential mode of vaccine development towards this gastric cancer. The aim of this study was to isolate and amplify the iceA gene from H. pylori directly using primers to amplify the iceA gene. To sequence, clone and express the gene of interest.

MATERIALS AND METHODS
Genomic DNA Isolation: Total genomic DNA from the bacteria was isolated by N-Cetyl-N, N, N-trimethyl-ammonium bromide (CTAB) method. The bacterial isolates were generously donated by the bacterial repository of Credora Life sciences, Bangalore, India. Total genomic DNA from the bacteria was isolated by N-Cetyl-N, N, N-trimethyl-ammonium bromide (CTAB) method described elsewhere (Wilson, K. 2001). In brief, the culture was centrifuged at 10000 rpm at 4°C and lysed with 675μl extraction buffer (100mM TrisHCl, 100mM EDTA, 1.4M NaCl, 1% CTAB and Proteinase K-0.03μg/μl). The suspension was incubated at about 37°C for 30 minutes. To the mixture 75μl of 20% SDS was added and incubated at 65°C for 2 hours. The suspension was then centrifuged and the supernatant was extracted with equal volumes of Chloroform and Isoamyl alcohol (24:1). The aqueous phase obtained after centrifugation was then extracted with 0.6 volumes of isopropyl alcohol. The mixture was allowed to stand undisturbed at RT for 1 hour. The suspension was then centrifuged again and the DNA was pelleted with 500μl of 70% ethanol. The DNA collected was then quantified using UV spectrophotometer (Shimadzu 1800 series).

PCR Amplification: The iceA gene was amplified by PCR using purified genomic DNA as a template. Oligonucleotide primers were synthesized to amplify the intact region of iceA gene. The forward primer, 5’ AGA GTT TGA TCC TGG CTC AG 3’ and the reverse primer, 5’- AAG GAG GTG ATC CAG CCG CA 3’, were purchased from Eurofins, Bangalore. These primers correspond to the gene iceA and thus the final PCR product was 1060 bp (Table 1). The PCR mixture consisted of 10x reaction buffer with MgCl2 (1.5mM), 2 L of dNTP mix (2.5mM), 2 L each of forward and reverse primers (10picomoles/1 each primer), 0.3 L of Taq DNA polymerase (5 U/ L), and 50ng/ L of template DNA in a total volume of 20 L. The PCR was performed with the following cycling profile: initial denaturation at 94°C for 2 min, followed by 30 cycles of 50s denaturation at 94°C, annealing at 51°C for 30s, and extension at 72°C for 1 min. The time for the final extension step was increased to 6 min. The PCR products amplified were then qualitatively analysed on 1% agarose gel. The PCR product was recovered using the QIA quick gel extraction kit, and the amplified product was then purified and used for cloning purpose.

Cloning of the iceA gene: The purified PCR product was ligated into the pTZ57R/T cloning vector (Fermentas, USA) and the resulting plasmid was transformed into the competent E. coli JM109. A 30 μl ligation reaction was performed using 1μl of the DNA template in a total reaction volume of 20μl. The reactions were performed in a Thermo cycler (G-Strom UK). Thirty amplification cycles were performed in the thermo cycler after initial DNA denaturation at 95°C for 5 min. Each cycle consisted of a denaturation step at 94°C for 50 s, an annealing step at 48°C for 40 s, and an extension step at 72°C for 1.30 min, with a final extension at 72°C for 6 min following the last cycle.

Cloning of the iceA gene: The purified PCR product was ligated into the pTZ57R/T cloning vector (Fermentas, USA) and the resulting plasmid was transformed into the competent E. coli JM109. A 30 l ligation reaction was setup in 3:1 molar ratio of insert and vector DNA as follows. 6 1 of PCR product (0.52pmol) was ligated with T4 DNA ligase (5Swiss units) and vector of concentration 50ng/ L was used. The total volume of the reaction mixture was made up to 30 l with nuclease free water (Sigma Aldrich). Ligation mixture was incubated at room temperature (25°C) for one hour after a short spin. The ligated product was then kept on ice until the transformation experiment started.

The competent cells were prepared using the protocol as described in Molecular cloning (Sambrook and Russel).
E. coli DH-5α was revived from the glycerol stock and inoculated into 5ml of Luria Bertani (LB) broth. The culture was incubated overnight at 37°C in an orbital shaker at 200rpm. The ligated product was mixed with 200μl of prepared competent cells and incubated on ice for 30 minutes without disturbing followed by heat shock treatment at 42°C for 2 minutes. The tubes were then incubated on ice for 2 minutes. To the treated cells 1ml of LB broth was added and the tubes were incubated 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 l of Ampicillin (50mg/ml) to a final concentration of 50 g/ml, 200 l of X-Gal to a final concentration of 80 g/ml and 20 l of IPTG to a final concentration of 80 g/ml was added. The incubated culture after 1 hour was centrifuged at 1000rpm for 10 minutes at room temperature and the pellet was resuspended in 100 l of fresh LB broth. From the suspension, 100 l was spread on LB agar plate. The plates were then incubated at 37°C overnight.

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.

 Confirmation of clones by restriction digestion: The purified plasmid was subjected to restriction digestion using restriction endonucleases (Merck, India). Restriction digestion was performed in 20 l reaction volumes with recommended units of enzyme and appropriate buffers at 37°C for 2 hours. The plasmid DNA (0.2 g/l) was double digested with restriction enzyme BamHI (10U/l) and EcoRI (10U/l). The contents of the mixture were mixed properly by pipetting and then the tubes were briefly spin down. The tubes were then incubated at 37°C for 2 hours. The digested samples were resolved on 1% low melting agarose gel for confirming the release of the insert by the restriction endonucleases. The released gene of insert was eluted from the agarose gel using gel extraction kit (Bioline USA).

Phylogenetic analysis: Phylogenetic tree were generated on the basis of sequences of iceA gene sequences using Clustal W 1.8. The variable and incomplete sites at both the 5' and 3' ends of the gene sequences were excluded from the alignment. Sites presenting alignment gaps were excluded from analysis. A rooted phylogenetic tree was constructed using the sequences reported here with Fig. 3: Showing the sequencing dendrogram

Fig 4: A tree plot was constructed with the NJ method using 1060bp fragment of the iceA gene showing the relationship of Helicobacter pylori 1989).
RESULTS AND DISCUSSION

Genomic DNA isolation and quantification: The Gram negative bacteria were cultured in the Brain heart infusion broth and genomic DNA was isolated by modified CTAB method. The isolated DNA was electrophoresed in 1% Agarose gel. The quantity and quality of DNA was analyzed by UV spectrophotometer.

PCR amplification of the iceA gene: Species specific primers were designed for the *Helicobacter pylori* using the sequences of iceA gene available in NCBI GenBank using Primer3 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 ~1060bp amplicon which shown in the figure 5.Epidemiological evidence showing an association of *iceA* strains with enhanced gastric inflammation and duodenal ulcer disease.

Cloning of PCR product in to T vector: PCR yielded a specific amplicon of 1060-bp in *Helicobacter pylori* strain. 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 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.1). The white colonies were picked up from the plates and cultured in Ampicillin containing LB broth.

Confirmation of clone by restriction digestion: The purified plasmid was subjected to restriction digestion using *BamH I* and *EcoR I*. After incubation at 37°C for 4 hours the restricted product was electrophoresed in 1% Agarose gel. The release of the gene product was visualized in the gel (Fig.2)

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, the sequences were compared to their corresponding GenBank sequences. The sequence had “perfect” match (similarity, 99%) with sequences of their corresponding gene (*iceA1*) from GenBank determined by using BLAST (version 2.7).

Cloning of *iceA1* gene into expression vector: The T vector clone was restricted with restriction enzyme (*EcoR I* and *NotI*) and the released gene product was gel purified using gel extraction kit. The purified gene fragment was quantified and ligated with linearized pET20b expression vector using T4 DNA ligase. The function of *iceA1*, whether it encodes a protein product and whether it plays a role in *H. pylori* pathogenesis, is unknown. Therefore, the goal of these studies was to define the transcriptional products of the *iceA1* locus to establish its potential for encoding functional proteins.
Inserted iceA1 gene was expressed significantly in the prokaryotic expression system, and specific strip at ~ 52 kDa was demonstrated in SDS-PAGE (Fig 5).

**SUMMARY AND CONCLUSION**

*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 helicobacter. 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 organism resistance. Because eradication modules have many 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 iceA gene from *Helicobacter pylori* to develop an antigen for the protective immunity against gastric helicobacter. To overcome the limits of antibiotic-based therapies, the vaccine approach has been undertaken since the last decade, leading us to identify some relevant bacterial antigens as candidates for vaccines. 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 confirmed by restriction digestion and sequence analysis. The sequence was found to be 99% similar to that obtained in GenBank. The gene was cloned into the expression vector and analysed on the SDS PAGE gel. After staining with coomassie a specific band was observed at an approximate molecular weight of 52KDa. Further study is required to go for a conclusion that the expressed protein will act as an antigen for the humoral immunity against the *Helicobacter pylori*. The experience from recent studies in our and other laboratories suggest that such vaccines should be given orally, ideally together with an effective mucosal adjuvant and provide protection against key pathogenic mechanisms of the bacteria.

The discovery of a protective antigen of *Helicobacter pylori* offers further hope that an effective vaccine can be produced for human usage. However, despite the fact that *H. pylori* a mucosal pathogen, protective immunity can be achieved by mucosal as well as parenteral administration of vaccines.

**REFERENCES**


