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Research Article

Cloning and Expression of *ice*A1 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 *ice*A (induced by contact with epithelium) is also proved to be an important factor for the pathogenecity. 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. i*ce*A gene was isolated and ligated into pTZ57R/T cloning vector. The ligated product was then cloned into DH5 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 gastritis[1,7], Several epidemiological studies have shown that *H. pylori* infection is also linked to severe gastritis-associated diseases[3,4] 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 diseases[2,3]. 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* strains[21].

Helicobacter pylori is a microaerophilic, gram negative spiral shaped bacterium that colonizes the human stomach[5,6]. It has been proved of its association with chronic gastritis, peptic ulcers and gastric cancer[8]. It has also been found to play a vital role in mucosa-associated lymphoid tissue lymphoma[13,14]. *WHO has assigned H. pylori* a definite class I carcinogen[16]. Even though the rate of prevalence of *H. pylori* is more in the developing countries [18,17] 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 environment[9]. The bacteria usually make the epithelial layers more susceptible to the acid attack, which in turn leads to severe inflammation[11]. This can be due to the involvement of several factors in the development of pathogeneicty. 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 pathogenecity[12, 18].

Induced contact with epithelium (iceA) gene has two alleles namely iceA1 and iceA2[17]. 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 ulcers[21,22]. But the iceA2 was found to be more prevalent in those patients who do not have any ulcers and are infected with *Helicobacter*[23].

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 pathogen[16, 19]. Due to the fact of its involvement in the contact to the epithelial layers, *ice*A 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 *ice*A gene from *H. pylori* directly using primers to amplify the *ice*A gene. To sequence, clone and express the gene of interest.

MATERIALS AND METHODS

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Table 1: Table showing the details of the forward and reverse primers designed towards amplification of the *ice*A gene of *Helicobacter pylori.*

Primer	Sequences (5'- 3')	GC%	Tm Value	Length	Product Size
FP	AGA GTT TGA TCC TGG CTC AG	50	51.8	20	~1060 bp
RP	AAG GAG GTG ATC CAG CCG CA	60	55.9	20	

Fig. 1: Blue white selection of the transformed bacterial cells in the Xgal-IPTG-Ampicillin-LB Agar

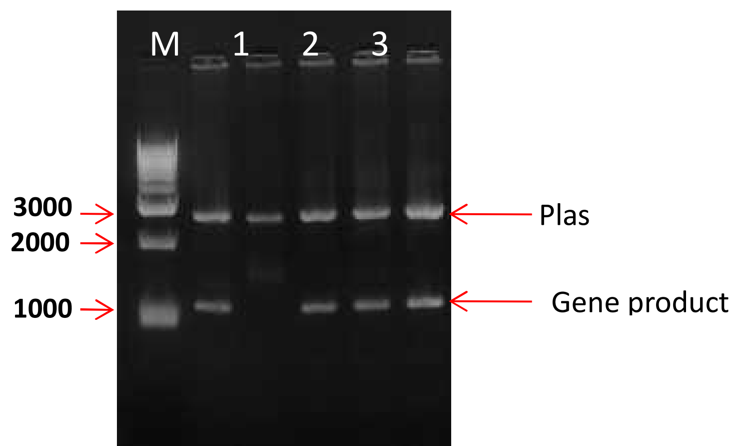

Fig.2: Restriction digestion of ligated plasmid using Bam H1 and EcoR 1

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 Tris HCl, 100mM EDTA, 1.4M NaCl, 1% CTAB and Proteinase K - 0.03µg/µl). The suspension was incubated at about 37^0C 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 1hour. The suspension was then centrifuged again and the DNA was pelleted with 500µl of 70% ethanol. The DNA collected was then quantified using UV spectrophotomer (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 *ice*A 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), 2µL of dNTP mix (2.5mM), 2µL each of forward and reverse primers (10picomoles/µl each primer), 0.3µL of Taq DNA polymerase (5 U/µL), and 50ng/µL of template DNA in a total volume of 20µL. 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 1min. 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.

Primer Details: The PCR assay 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 *ice*A 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 30µl ligation reaction was setup in 3:1 molar ratio of insert and vector DNA as follows. 6µl of PCR product (0.52pmol) was ligated with T4 DNA ligase (5weiss units) and vector of concentration 50ng/µl was used. The total volume of the reaction mixture was made up to 30µl 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 later kept on ice until the transformation experiment started.

The competent cells were prepared using the protocol as described in Molecular cloning (Sambrook and Russel,

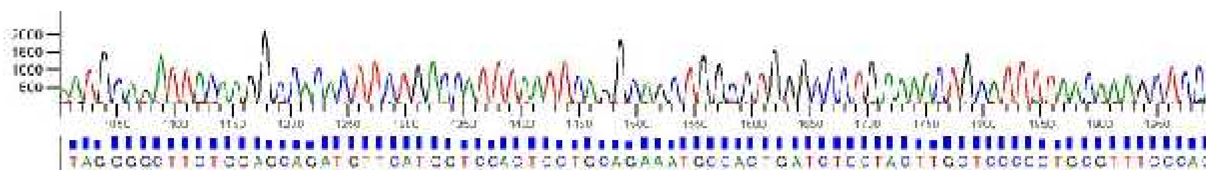

Fig. 3: Showing the sequencing dendrogram

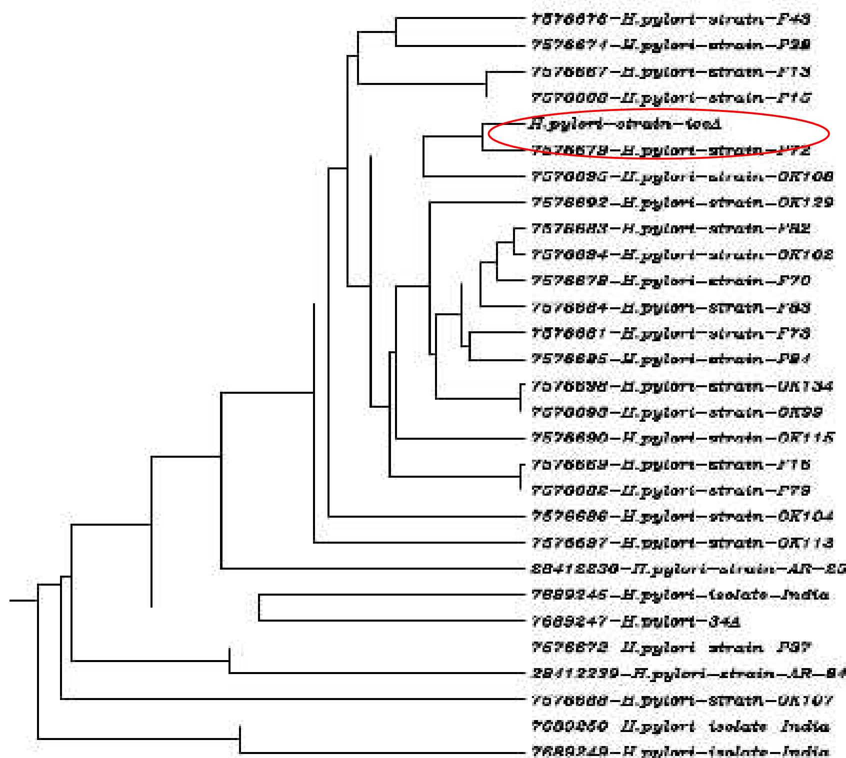

Fig.4: A tree plot was constructed with the NJ method using 1060bp fragment of the iceA gene showing the relationship of Helicobacter pylori

1989). *E. coli* DH-5- 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 2minutes. 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, 50µl of Ampicillin (50mg/ml) to a final concentration of 50µg/ml, 200µl of X-Gal to a final concentration of 80µg/ml and 20µl of IPTG to a final concentration of 80µg/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 100µl of fresh LB broth. From the suspension, 100µl 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 tranformant. 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 20µl reaction volumes with recommended units of enzyme and appropriate buffers at 37°C for 2hours. The plasmid DNA (0.2µg/µl) was double digested with restriction enzyme *Bam*HI (10U/µl) and *Eco* RI (10U/µl). 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

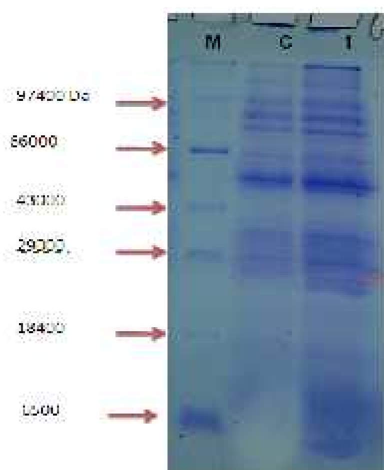

Fig.5: SDS PAGE analysis of iceA gene expression (M- Protein Molecular weight ladder (Phosphorylase B-97400, Bovine Serum Albumin-66000, Ovalbumin-43000, Carbonic Anhydrase-29000, Lactoglobulin-18400, Aprotinin-6500) C- Control, 1- iceA gene expression)

diverse *Helicobacter pylori* sequences previously deposited in the GenBank database.

Gene cloning into expression vector: Vector pET20b (50ng/µl) together with eluted gene of insert (0.52pmole) was added to the ligation mixture and then incubated. The pTZ57R/T-*iceA* and pET20b (+) were digested by restrictive enzymes with *Bam*H I and *Eco*RI. The inserted fragment of pTZ57R/T-*iceA* was collected from electrophoretic gel, and then it was ligated with the linearized pET20b (+) by T4 ligase at 27^0C for 3 hours. The recombinant was transformed into *E. coli*DH5 by $CaCl_2$ method described earlier, selected from agar plate containing Ampicillin (50mg/ml) and confirmed by restriction enzyme mapping.

Protein extraction: The bacterial cells were harvested by centrifugation (7,000 x g) at 4°C and washed with sterile distilled water. The cells were then suspended in 10 ml of ice-cold acetone (analytical grade), and allowed to stand on ice for 5 min. the suspension was then centrifuged (7,000 x g) at 4°C. Residual acetone was removed by air drying, and the proteins were then extracted by incubating with 1.0 ml of 1% sodium dodecyl sulfate (SDS) for 2 min. The protein concentration was estimated at 280nm using UV spectrophotomer (Shimadzu 1800 series). From the stock 1µl Protein was mixed with 99µl sterile distilled water to get 100 times dilution.

The collected protein fractions in sample buffer (10% SDS, 10mM Dithiothreitol, 20% Glycerol, 0.2M Tris-HCl and 0.05% Bromophenol blue) were then separated on SDS PAGE electrophoresis containing 5% stacking gel and 12% resolving gel. The gel was removed from the plates carefully and stained using Coomassie Brilliant Blue dye, by agitating slowly on a rocker, overnight and subsequently destained for a few times until protein bands were visualized. The molecular weight of protein bands were determined by comparing them with the molecular weight markers.

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 *ice*A gene: Species specific primers were designed for the *Helicobacter pylori* using the sequences of *ice*A gene available in NCBI GenBank using Primer3 Software. The predicted primers were validated initially *in silicon* and subsequently in wet lab. The primers could yield an amplicon of the expected size specific to *ice*A 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 *iceA1* 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 DH5 . The transformation was done by heat shock method and transformed cell was cultured in the Xgal-IPTG-Ampicillin-LB Agar plate at 37ºC 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 *Bam*H1 and *Eco*R 1 (Merck, India). After incubation at 37ºC for 4 hours the restricted product was electrophoresed on 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 *ice*A 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 *ice*A 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 (*ice*A1) from GeneBank determined by using BLAST (version 2.7).

Cloning of *iceA1* gene into expression vector: The T vector clone was restricted with restriction enzyme (*Eco*R1 and *Not*1) 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 *ice*A1 locus to establish its potential for encoding functional proteins.

Inserted *ice*A1 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 *ice*A 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 *ice*A2 gene from *Helicobacter pylori* was amplified using specific primers and cloned in pTZ57R/T and transformed into DH5 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.

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