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CLONINIG AND EXPRESSION OF *CAG* PATHOGENICITY ISLAND ANTIGENS FOR THE PRODUCTION OF VACCINE AGAINST *HELICOBACTER PYLORI*, THE RISK FACTOR FOR GASTRIC CANCER

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ABSTRACT

In the present study we cloned and characterized the *cagN* gene from *Helicobacter pylori* to develop an antigen for the protective immunity against gastric helicobacter. The *cagN* 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 *cagN* gene amplified by the specific primer is closely matched (99%) with a *Helicobacter pylori* strains. The gene restricted from the cloning vector was ligated to expression vector. Transformation was confirmed with plasmid extraction and followed by restriction digestion. IPTG was used as an inducer for the expression of cagN protein and the protein was successfully isolated and quantified. The quantified protein was subjected to SDS PAGE to evaluate the expression of that protein. After staining with coomassie staining a specific band was observed at an approximate molecular weight of ~20 KDa. The discovery of a protective antigen of *Helicobacter pylori* offers further hope that an effective vaccine can be produced for human usage.

KEYWORDS: ***Helicobacter Pylori, Cag,*** **Gastric Cancer, Cloning and Expression**

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INTRODUCTION

Helicobacter pylori is a gram negative, microaerophilic bacterium that can inhabit various areas of the stomach, particularly the antrum. It causes a chronic low-level inflammation of the stomach lining and is strongly linked to the development of duodenal and gastric ulcers and stomach cancer[1].Since the introduction of *Helicobacter pylori* to the medical community by Marshall and Warren almost two decades ago, *Helicobacter pylori* has been the focus of basic biochemical and clinical research and debate. Its relevance to human disease, specifically to peptic ulcer disease, gastritis, and gastric malignancy, is indisputable. Many questions, however, still remain concerning the optimal diagnostic and therapeutic regimens with which to approach the organism[2]. The *cag* pathogenicity island (PAI) has about 30 genes, part of which code for a complex type IV secretion system. The low GC-content of the *cag* PAI relative to the rest of the *Helicobacter* genome suggests that the island was acquired by horizontal transfer from another bacterial species[3]. Over half of the *H. pylori* strains in Western countries are thought to carry the *cag* PAI[4]. Not only do these strains create a stronger inflammatory response in the stomach but they also create a greater risk for developing ulcers or cancer than the strains lacking the *cag* PAI[5]. The *cag* PAI expresses a type IV secretion system after the attachment of the bacterium to the epithelial cells of the stomach. This system inserts peptidoglycan from the bacterial cell wall into the epithelial cells. This peptidoglycan acts as an inflammatory inducing agent within epithelial cells. It is recognized by the cytoplasmic immune sensor Nod1 that stimulates the expression of cytokines which promote inflammation [6]. The type IV secretion system also injects the *cag* PAI-encoded protein *cagA* into the stomach's epithelial cells, where it disrupts the cytoskeleton, adherence to adjacent cells, intracellular signaling, cell polarity and other cellular activities[7].in ordfer to functyionally aqctive in the cell the *CagA* protein were phosporilated by thyrosine kinas. Activation of the EGFR by *H. pylori* is linked with altered signal transduction and gene expression in host epithelial cells that may contribute to pathogenesis. It has also been suggested that a c-terminal region of the *cagA* protein (amino acids 873–1002) is able to regulate host cell gene transcription independent of protein tyrosine phosphorylation[8,9]. Treatment of *Helicobacter pylori* infection can lead to exacerbation of GERD in many patients, prompting many gastroenterologists to defer endoscopic anural biopsies in patients with significant GERD and absent ulcer. Conversely, other studies using endoscopic findings, pH probe measurements, and histology to determine the presence of *Helicobacter pylori* did not find any association between GERD (in any of its manifestations) and infection with *Helicobacter pylori*. Clearly, more definitive studies are necessary to define the relationship, if any, between these 2 entities[10]. Research study based communication found that licorice extracts are also effective against *Helicobacter pylori* strains that are resistant to both amoxicillin and clarithromycin, making them viable as chemo preventive agents for peptic ulcer or gastric cancer in *Helicobacter pylori* infected individuals[11].

MATERIALS AND METHODS

Bacterial culture and media preparation

Helicobacter pylori isolate was taken from the bacterial repository of Credora Life sciences, Bangalore, India and inoculated in Brain Heart infusion broth. The inculcated broth was incubated at 37^{o}C in microaerophilic condition in an anaerobic chamber (Himedia, India) with 20% CO2 according to manufacturer's instructions.

DNA Isolation and PCR

DNA was isolated from blood samples using Qiagen DNA Mini Kit as per the manufacturer's instructions. The PCR was performed using the following conditions: complete denaturation (95°C for 3 min), followed by 30 cycles of amplification (94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min) and the final elongation step (72°C for 2 min). PCR products were separated on 1 % Agarose gel.

Cloning and Plasmid Isolation

The PCR product was eluted from the gel and cloned in ligate cloning vector (pTZ57R/T) as per manufacturer's instructions. The positive clones were picked from antibiotic plate and colony PCR was performed for confirmation of cloning. From the transformed cells Plasmid was isolated by alkaline lysis method[12]. and electrophorized on 1% Agarose gel. The purified plasmid was subjected to restriction digestion using *Bam*H1 and *Eco*R1. The release of the gene product was checked on 1% agarose gel.

Sequencing and Data Analysis and Gene Expression Analysis

Eluted the gene product was sequenced, and ligated with the expression vector pET 20b, then Ligated plasmid was transformed in to the bacterium DH5α cells as described earlier and Plated on LB agar- Ampicillin plates. The white colonies from the LB- Ampicillin agar plates were inoculated in LBAmpicillin broth and incubated at 37°C for overnight and plasmid was isolated by Alkaline lysis method described earlier. The transformed bacteria were inoculated in 50 ml of LB broth containing IPTG (0.5mM) as inducer for the expression of the gene. *E.coli* strain without plasmid was used as a control. The inoculated culture flask was incubated at 37°C at 150 rpm for 24 hours in an orbital incubator shaker.

Protein extraction

The bacterial cells from 50 ml culture were harvested by centrifugation (7,000 x g) at 4°C, washed with sterile distilled water. The cells were then suspended in 10 ml of ice cold acetone (analytical grade), allowed to stand on ice for 5 min, and collected by centrifugation (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.

Protein estimation and SDS PAGE

The protein concentration was estimated at 280nm using UV-VIS spectrophotomer (Vivaspec Biophotometer, Germany). From the stock 1µl Protein was mixed with 99-µl sterile distilled water to get 100 times dilution. The diluted protein concentration was directly estimated by Vivaspec Biophotometer. The SDS PAGE experiment was designed to separate a protein based on molecular weight compared with control. Samples were prepared and gel was made to run according to manufacture instructions.

RESULTS AND DISCUSSION

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 electrophorized in 1% Agarose gel (Fig.1).

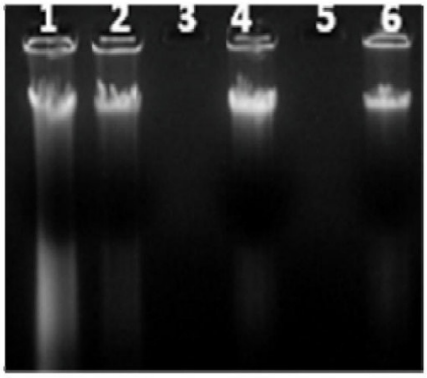


Fig. 1. Genomic DNA isolated from Helicobacter pylori (Line 1, 2, 4, 6- Genomic DNA)

The specific primers were designed using Primer3 Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) and the designed oligonucleotides were synthesized in Sigma Corporation USA. The primer detail is mentioned in Table 1.

Primer	Sequences (5'- 3')	Tm Value	Length	Product Size
FP	TGA ATG ACA GCC CTA ATG GCA	52.4^{0}C	21	642 bp
RP	CTG AGA TGA CAA GCT ATG AT	51.4^{0}C	20	

Table.1. Primer Details

The primers were found to produce ~2112 bp amplicon which shown in the (Fig.2).

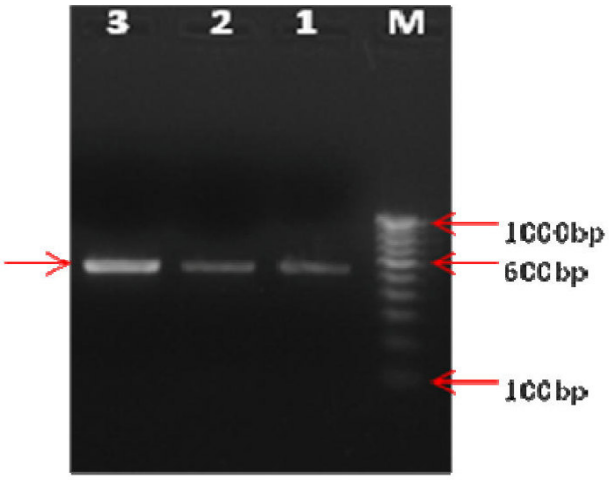


Fig. 2. PCR amplification of *cagN* gene by specific primers (M- 100bp ladder, Line 1, 2, 3, -*cag*N gene product)

PCR yielded a specific amplicon of 612-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 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 37oC for overnight (Fig.3).

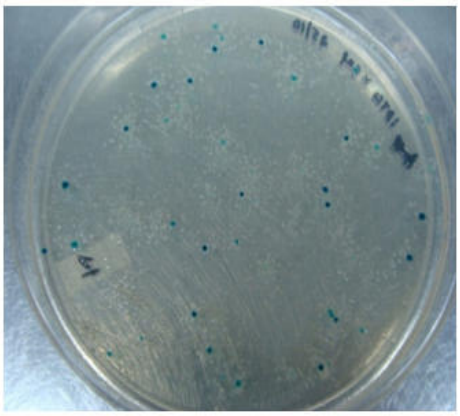


Fig. 3. Blue white selection of the transformed bacterial cells in the Xgal-IPTG-Ampicillin-LB Agar

Plasmid was isolated from the transformed cells and was electrophorized on 1% Agarose gel (Fig. 4).

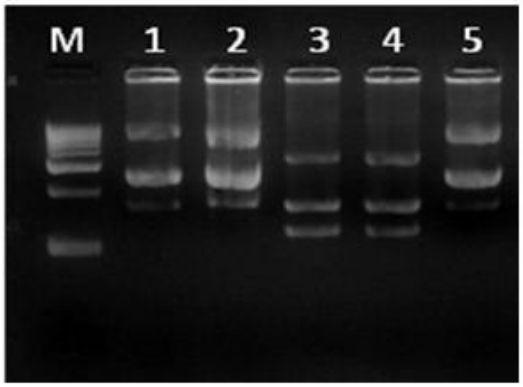


Fig. 4. Plasmid isolated from the transformed bacterial cells

The purified plasmid was subjected to restriction digestion using *Bam* H1 and *Eco*R 1 (Merck, India). After incubation at 37°C for 4 hours the restricted product was electrophorized on 1% Agarose gel. The release of the gene product was visualized in the gel (Fig. 5)

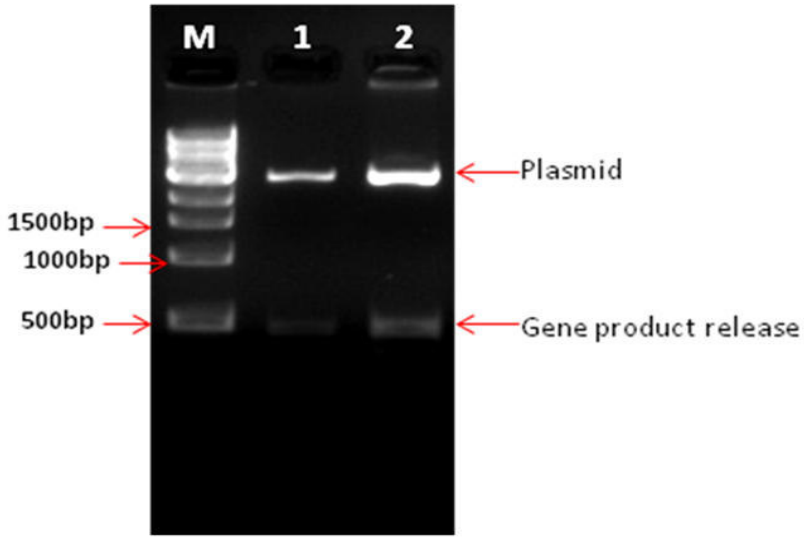
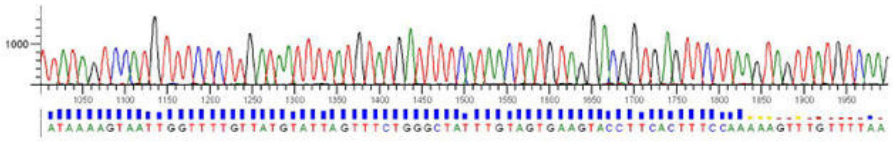


Fig.5. Restriction digestion of ligated plasmid using *Bam* H1 and *Eco*R 1

The gene was identified by sequencing of plasmid. An approximately 612-bp region of the *cag* gene was sequenced (Table3) at Eurofins, Bangalore. The sequence data was shown bellow**.** Nucleotide sequence analysis of gene was used to investigate the identity of bacterial *cagN* 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 (*cagN*) from GenBank as determined by using BLAST (version 2.7).



> *cagN* gene sequence

5′ TGAATGACAGCCCTAATGGCAATTCTTCCACTGAAACCAAATCTAATAAAGATGATAACTTTGATGAG
ATGATCAATAAGGTGAATGAAGCTTTTGTGAAACCTGCTGCTCCGCTTGTGCCTGATGAGTGGAGAACGC
CTGAAATTGAAATCGTTATCAATAAGTGTATTATTTCAAGCAACGATTATGATGGGTTAAGAAAGTGTTT
GATCAAAGACATCAAGGATCAAAAAATTCTTGCCCCCTTATTAGAAAAAATTCAAGAAATAGAGACAGAA
AATAACAAGTTTTCTAGACAACACTTGAGTGGTTTAAAACTCGCTCTTAATAACAGCAACAATAGAACCT
TTCTTATAGCTTCGTGCGCTATTTGTGAGAAGAGAAAAAAAGAAATGGAGCAAGAAAATAACTACCAAGA
TACTACAAACGCAAGTGAGTTTGGAGTTACTGATACAAAAGAAAATGAAGCAAAAGATGCAACATTCTCA
AACAATCGCTCTAAATCCGAACTGCCCAATAGCGTCATTAATCAAATAGAACAAAGCATCGCTCATGGAA
AAAAATAGCGATCCAAATTATTAGCTCAAAAAACAACTAGAGAAACAAATCCCAAAAATGAGAAATCATA
GCTTGTCATCTCAG 3′

Table.2: The N-J tree with branch length was plotted using ClustalW sequence alignment (http://align.genome.jp/), showing the relationship of *cagN* gene among the closest *Helicobacter pylori strains* in the NCBI database (Fig.6)

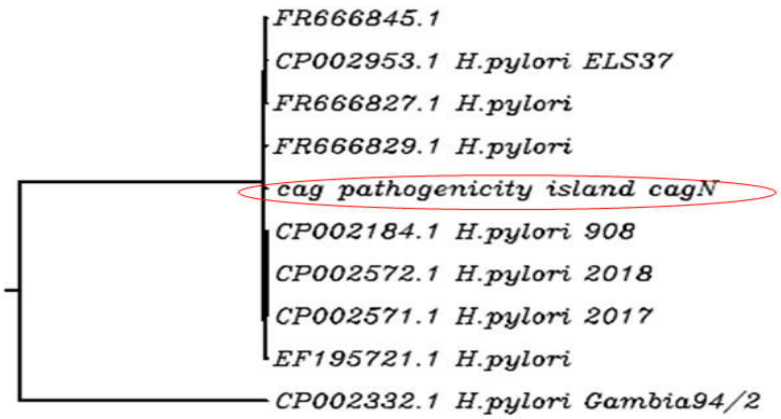


Fig.6. A tree plot was constructed with the NJ method using 600 bp fragment of the *cagN* gene showing the relationship of *Helicobacter pylori*.

The T vector clone was restricted with restriction enzyme (*Eco*RI and *Bam*H1) and the released gene product was gel purified using a gel extraction kit. The purified gene fragment was quantified and ligated with linearized pET20b expression vector (Novagen, Germany) using T4 DNA ligase. Inserted *cagN* gene was expressed significantly in the prokaryotic expression system, and specific strip at ~ 20 KDa was demonstrated in SDS-PAGE (Fig.7). In order to analyse the pathogenicity of *H. pylori* which were transformed from spiral form by exposed to antibiotic in and analyzed the protein expression of their respective genes. As the results, protein with the molecular weight over *Mw* 74000 decreased. To clonclude the virulence of *H. pylori* non of there individual

genes were deleted, how ever only the molecular weight over *Mw* 74000 in coccoid *H pylori* decrease which suggested that *H. pylori* may have potential pathogenicity

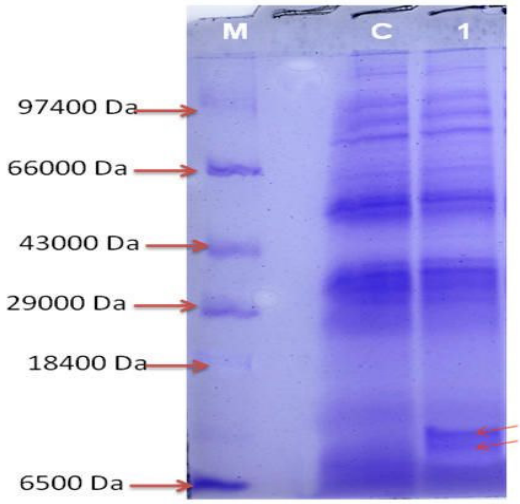


Fig.7. SDS PAGE analysis of *cagN* gene expression [M- Protein Molecular weight ladder, (Phosphorylase B-97400, Bovine Serum Albumin-66000, Ovalbumin-43000, Carbonic Anhydrase-29000, Lactoglobulin-18400, Aprotinin-6500) C- Control, 1- *cagN* gene expression)

However, in Asia, most of the *Helicobacter pylori* strains are *cag* positive, irrespective of clinical outcome. Thus, conclusions about the relationship between *Helicobacter pylori* genotypes and clinical outcome derived from one geographic region may not be true for other geographic regions. The relationship between *cag*-positive *Helicobacter pylori* and an increased risk of developing clinical outcomes is controversial[13], because even in existing animal models of *H. pylori* infection, the importance of the *cag* PAI has been controversial[14]. The origin of the *cag* PAI, which was acquired by horizontal transfer, is unknown, and most of the proteins it encodes are novel and have no known function. It is possible that many of these genes served functions in the bacterium from which the PAI originated and that they are not important in the context of *H. pylori* infection and disease outcome. Alternatively, because *cagN* has been retained by *cag* PAI strains and is generally conserved[15], it may provide a fitness advantage to H. pylori during some aspects of its infectious cycle that we cannot readily detect in cell culture Moreover, it is relatively difficult to detect the *cag* gene by PCR with a single primer pair due to high homology between the sequences of other *cag* family genes.

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